

68 to 1

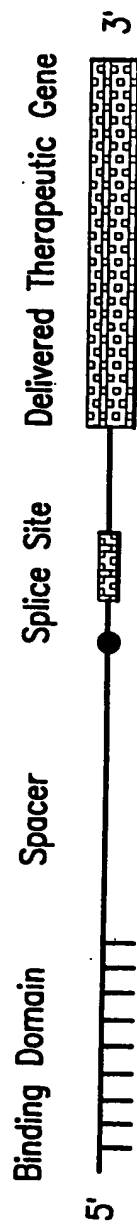


FIG. 1A

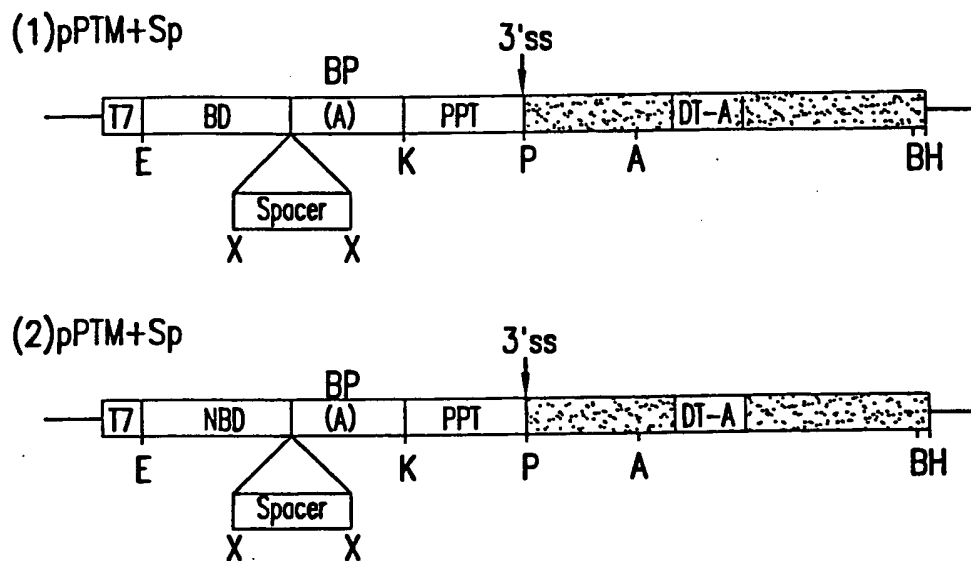


FIG.1B

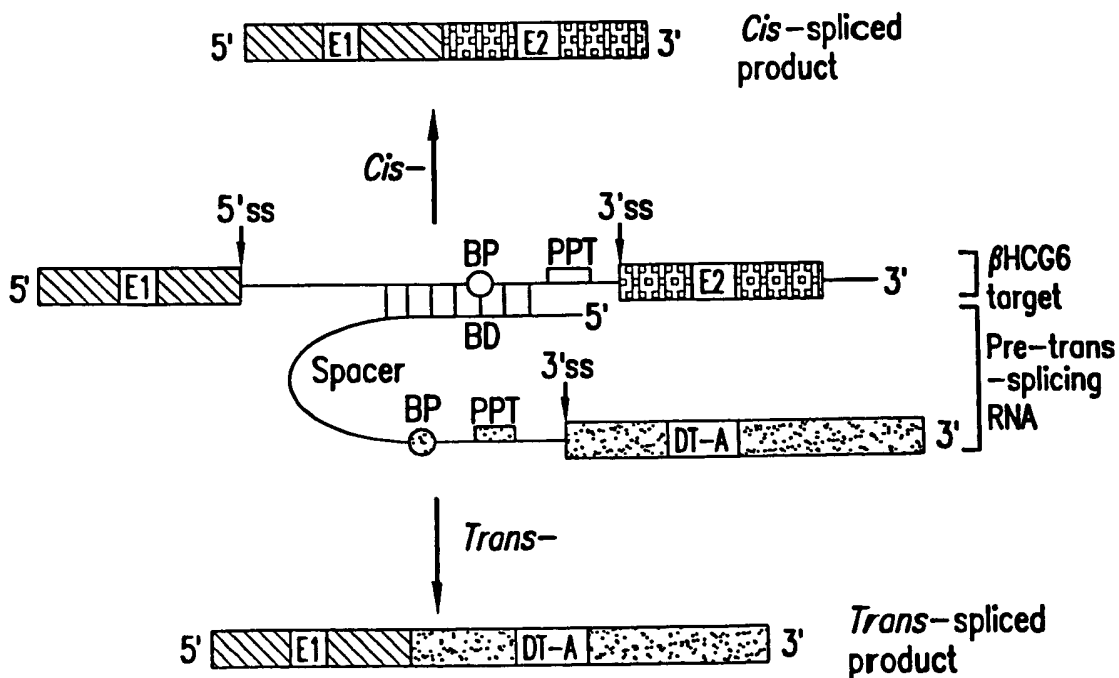


FIG.1C

05443492-03601

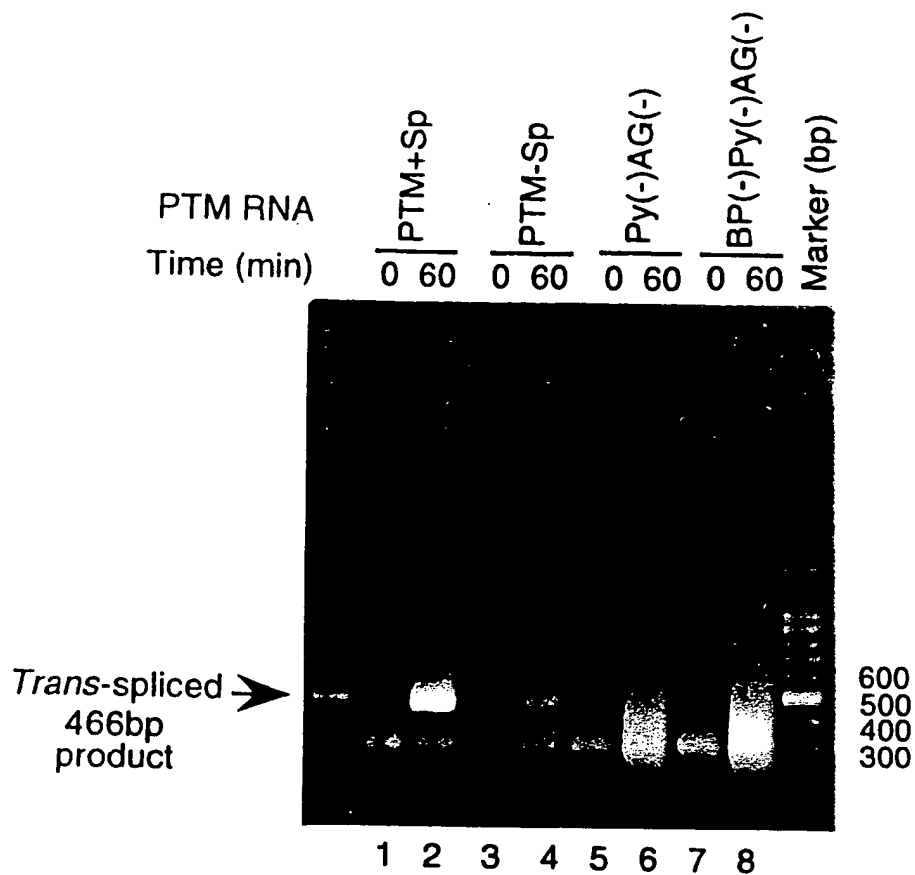
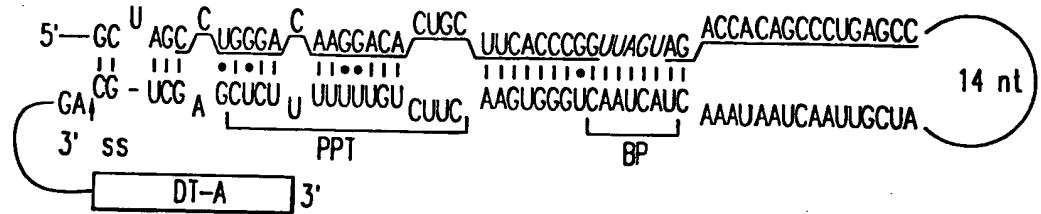
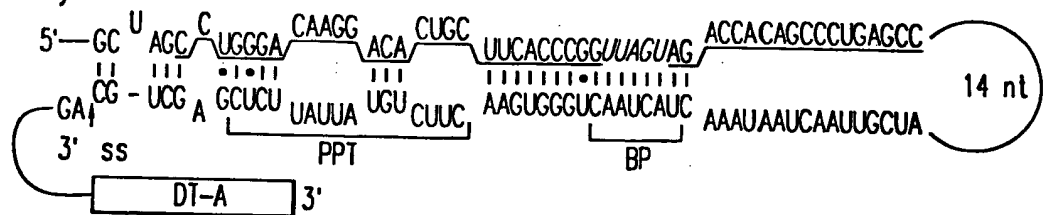


FIG.2A

1. PTM+SF:



2. PTM+SF-Py1:



3. PTM+SF-Py2:

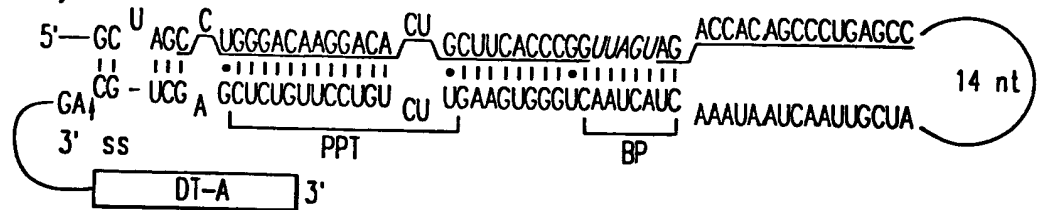


FIG.4A

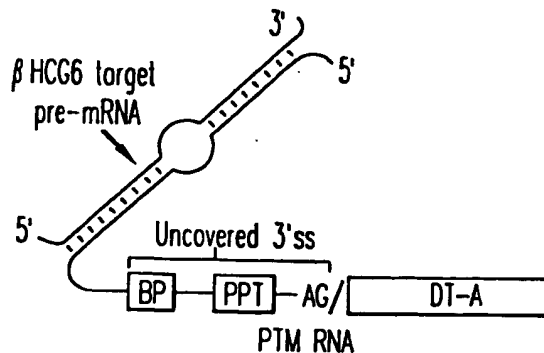


FIG.4B

105290"264T460

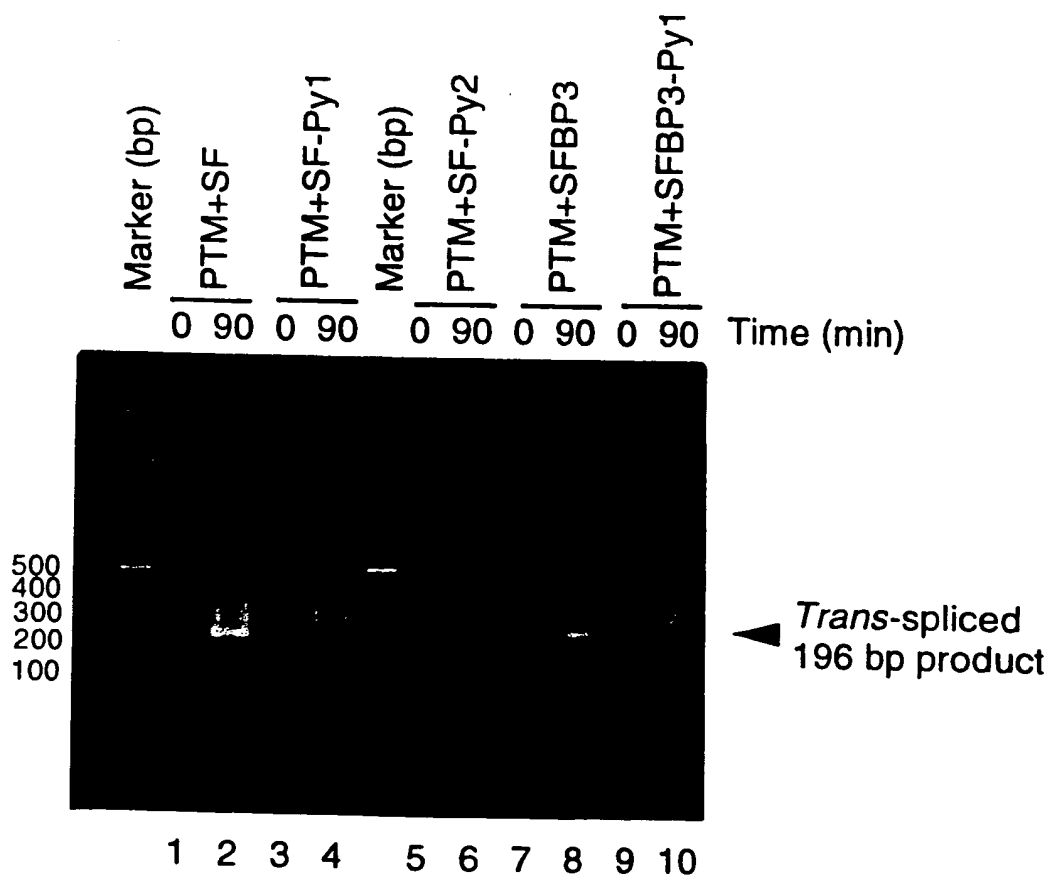


FIG.4C

Linear PTM				Safety PTM				Forward Primer	
Marker (bp)				βHCG-F	β-globin-R	βHCG-F	β-globin-R		
				HCGR2	DT-3R	HCGR2	DT-3R		
				DT-3R	β-globin-R	DT-3R	β-globin-R		
				β-globin-R	HCGR2	β-globin-R	HCGR2		

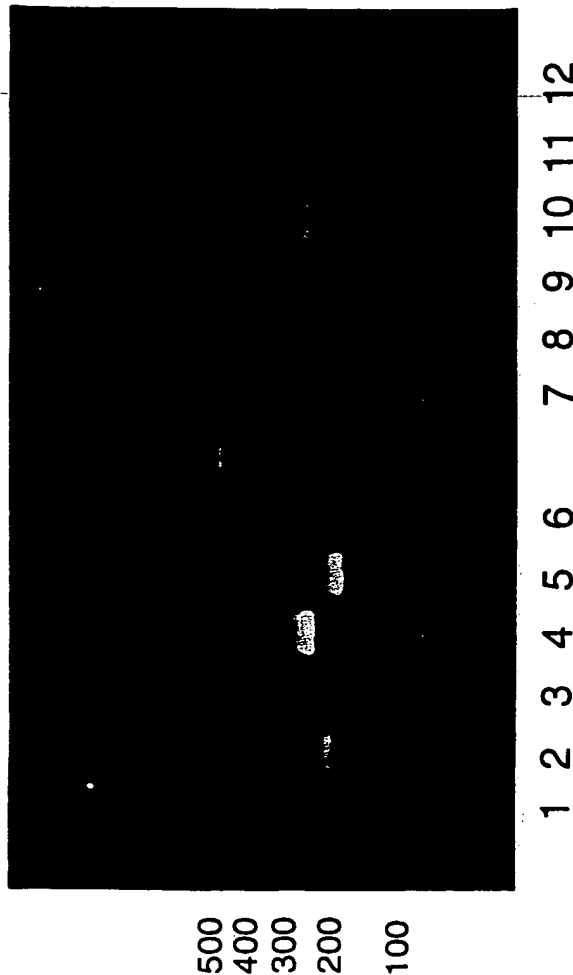


FIG.5

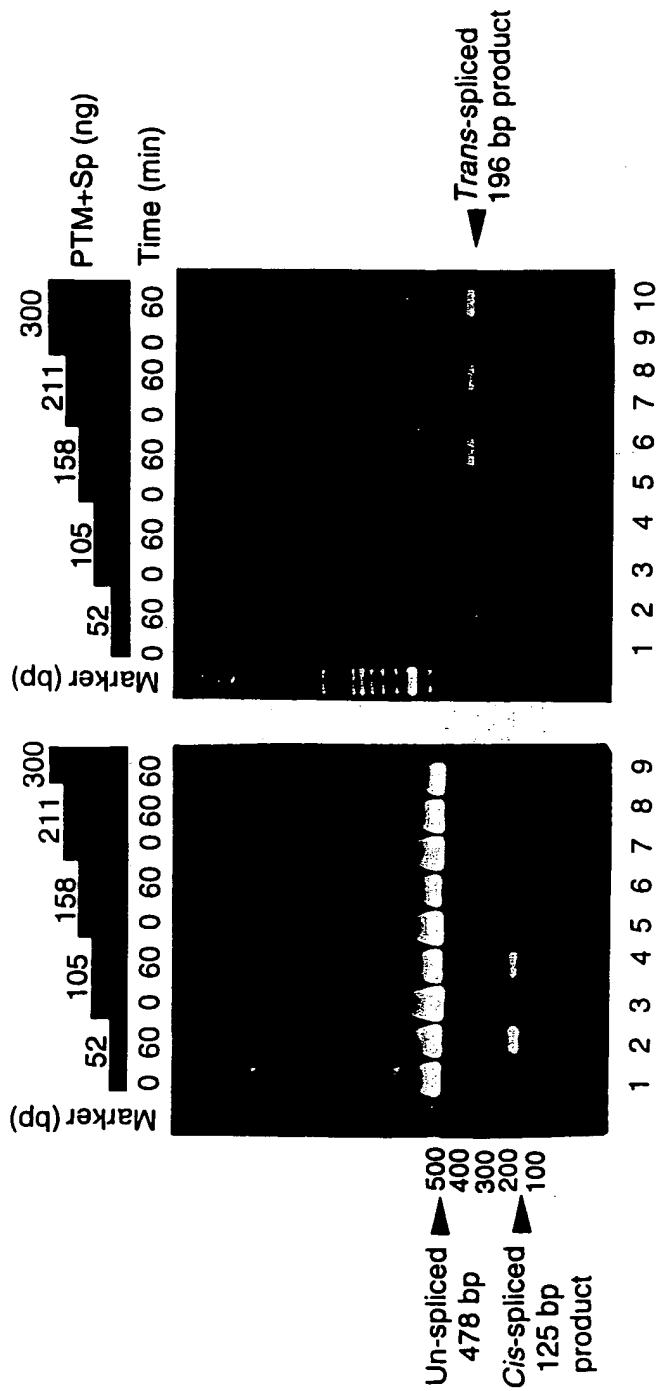


FIG. 6A

FIG. 6B

68 fo b

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105290" 264460

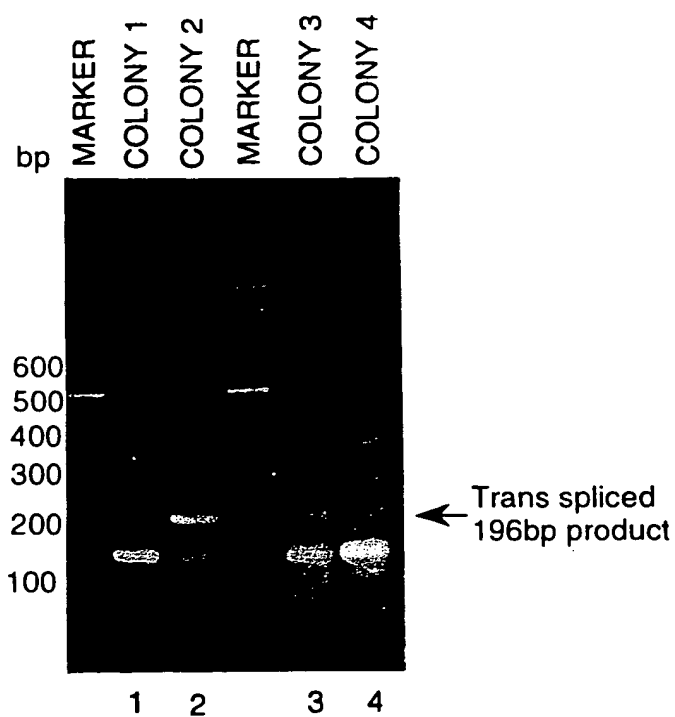


FIG.7A

68 80 11

EXON 1 OF β HOG6 ↓
 5'-CAGGGACGCACCAAGGATGGAGATGTTCCAG-GGGCTGATGATGTTGTT
 ↓ 1ST CODING NUCLEOTIDE OF DT-A
 GATTCCTTAAATCITTTGTGATGGAAAACITTTCTTCGTACCAACGGGACTA
 AACCTGGTTATGTAGATTCATTCAAAA-3'

FIG.7B

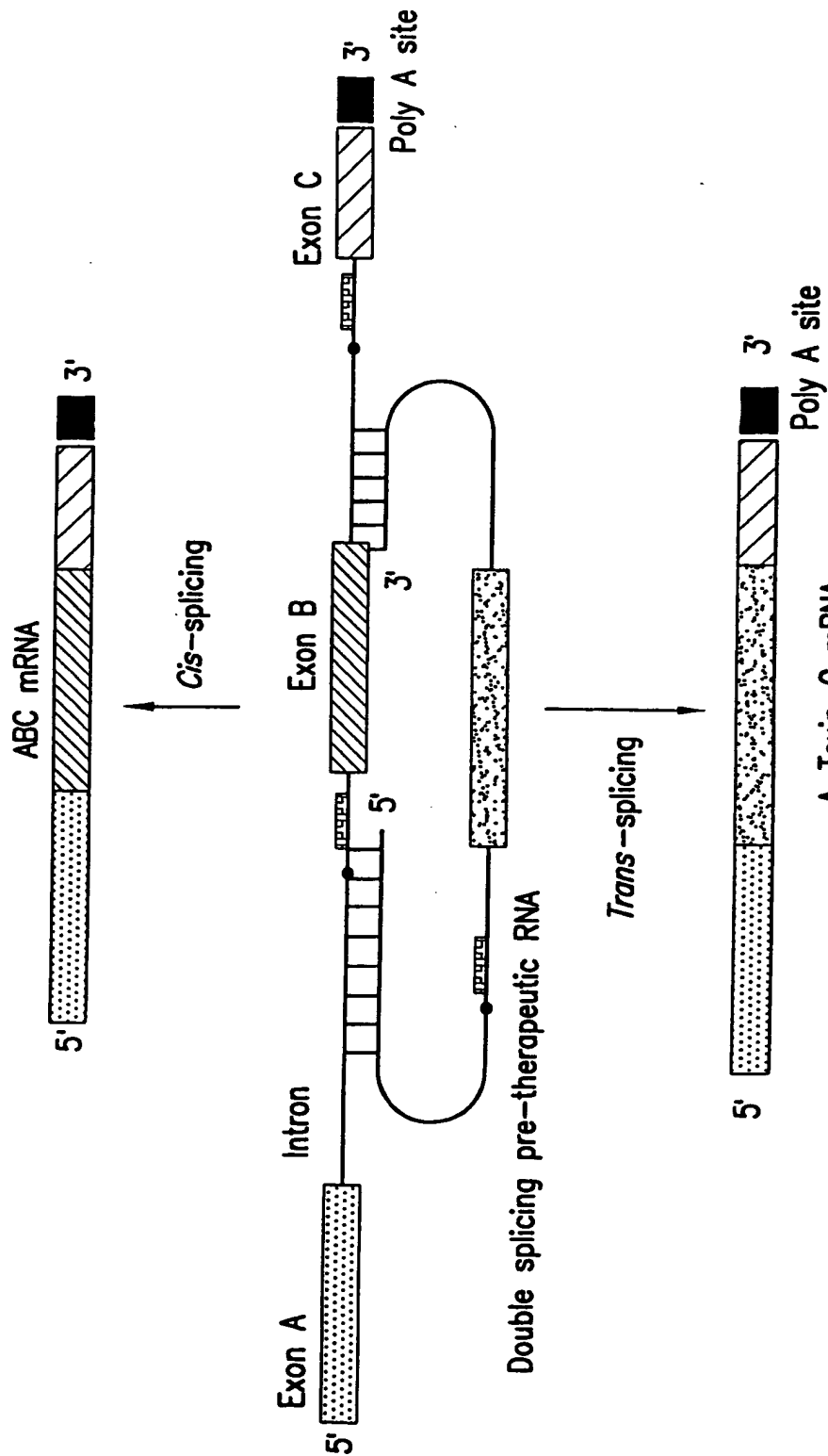
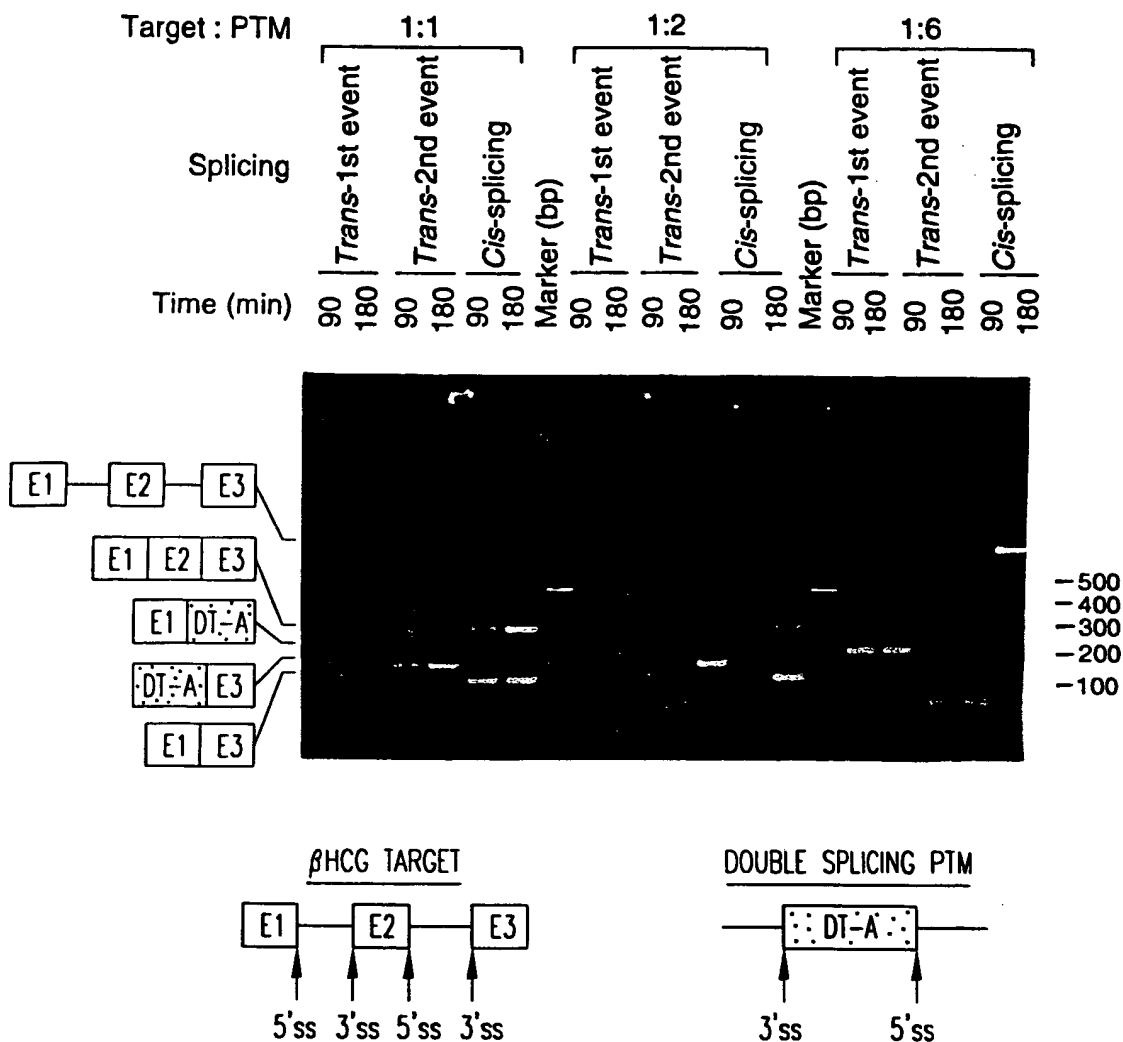


FIG.8A



Cis-spliced products

E1 E2 E3 = NORMAL *cis*-SPLICING (277bp)

E1 E3 = Exon SKIPPING (110bp)

Trans-spliced products

E1 DT-A = 1st EVENT, 196bp. *Trans*-SPLICING BETWEEN 5' ss OF TARGET & 3' ss OF PTM.

DT-A E3 = 2nd EVENT, 161bp. *Trans*-SPLICING BETWEEN 3' ss OF TARGET & 5' ss OF PTM.

FIG.8B

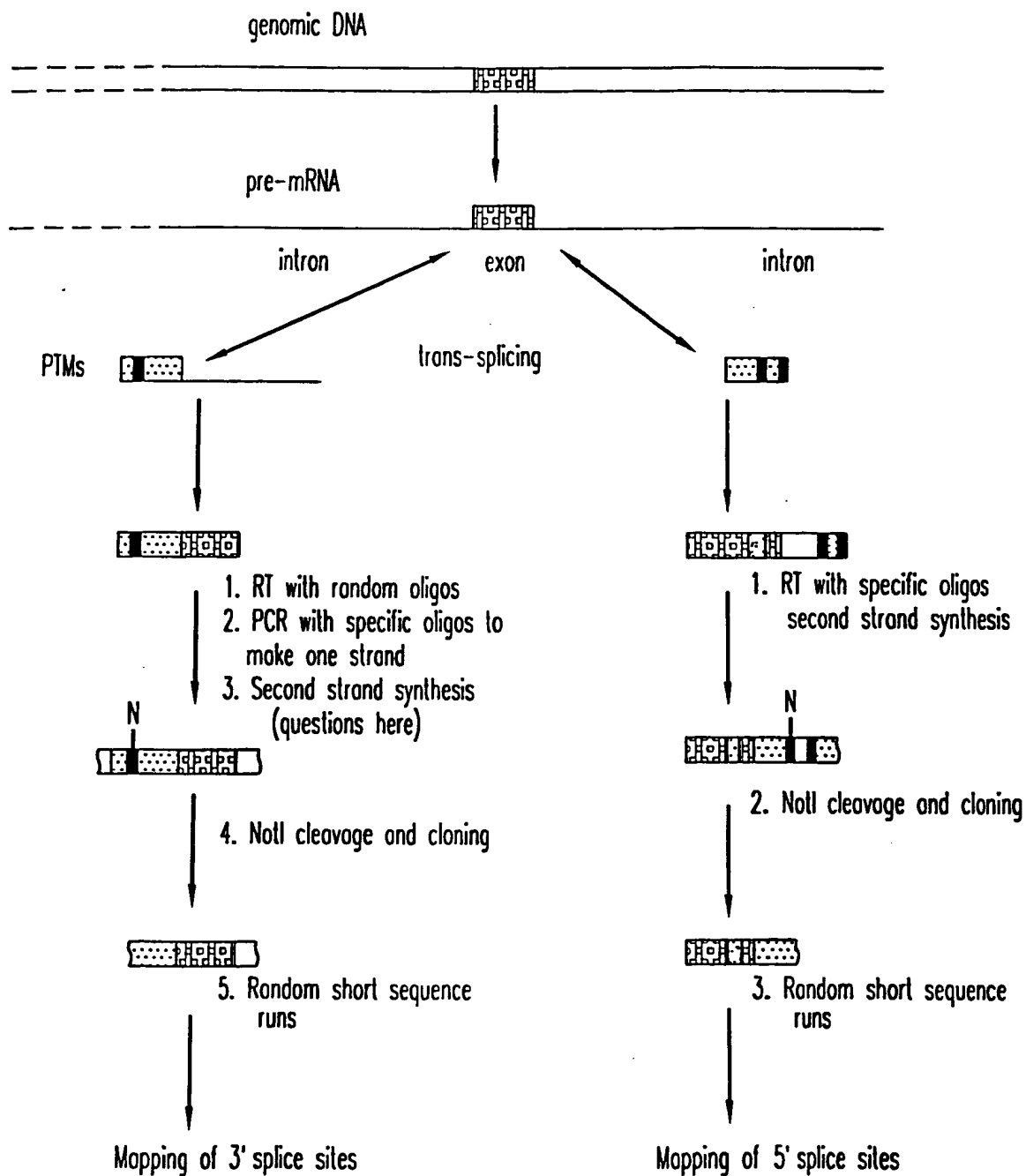
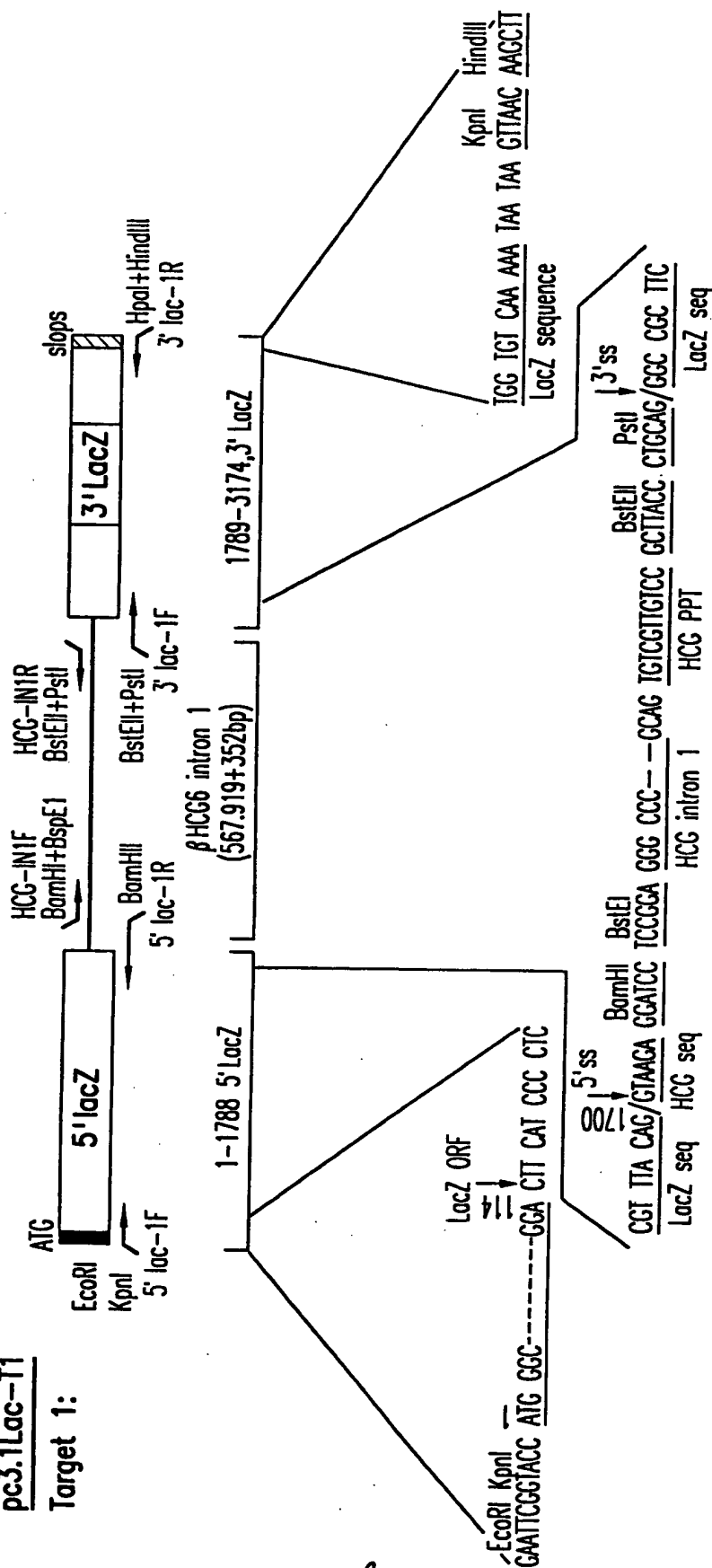


FIG.9

pc3.1Lac-T1

Target 1:



PTMs

pc3.1PTM2:

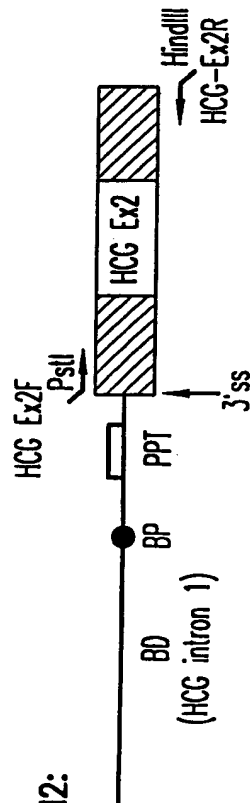


FIG.10A

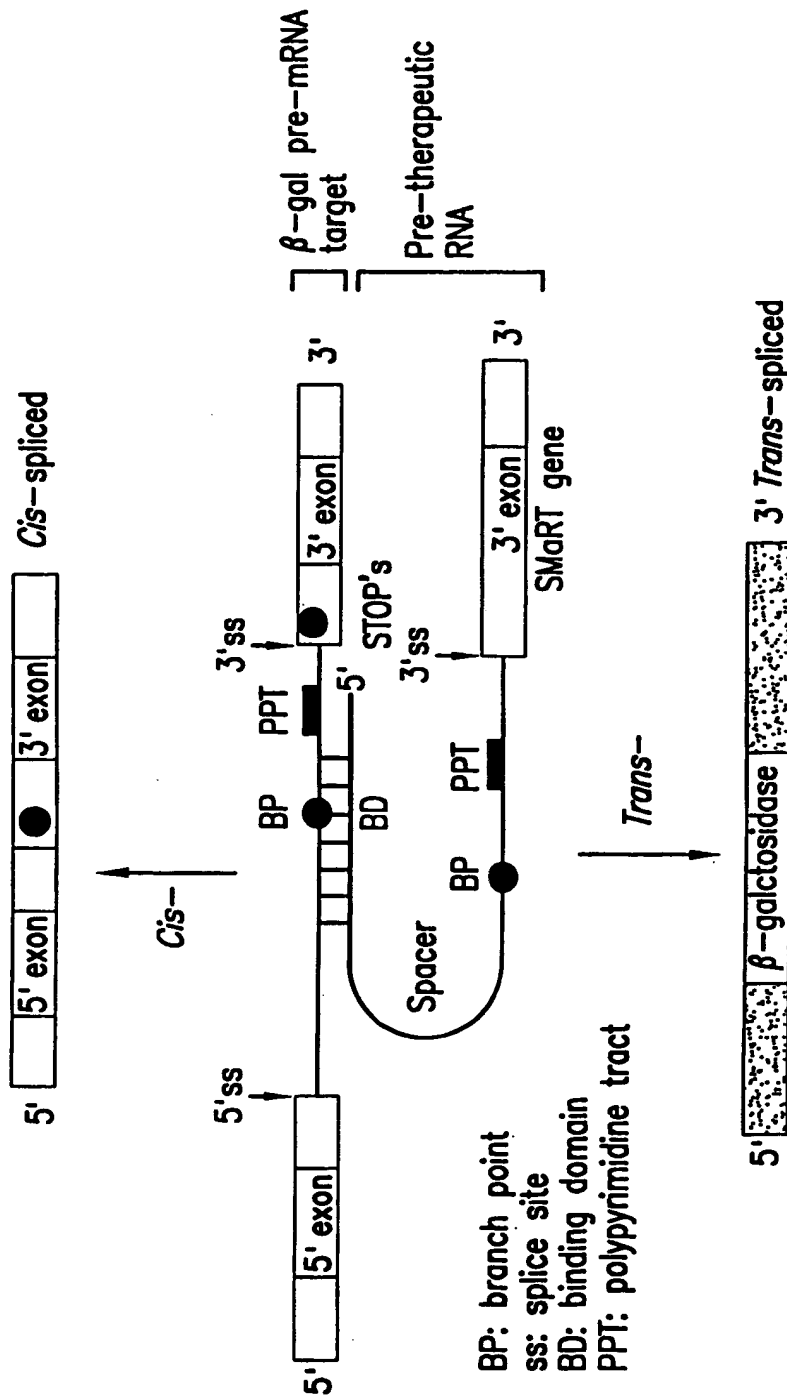


FIG.10B

80 21

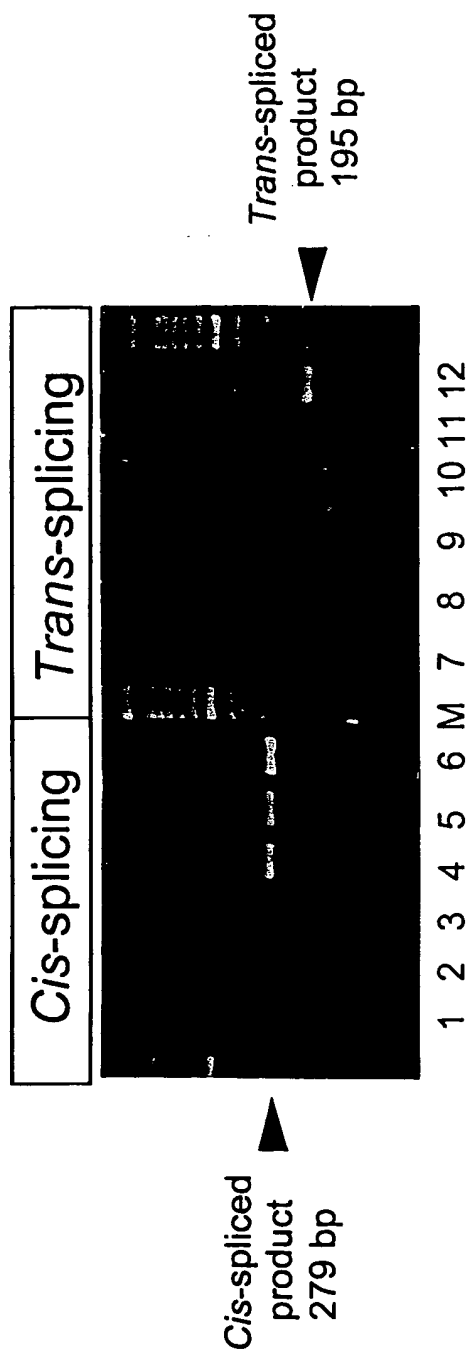


FIG.11A

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094149.082901
105280" 254460

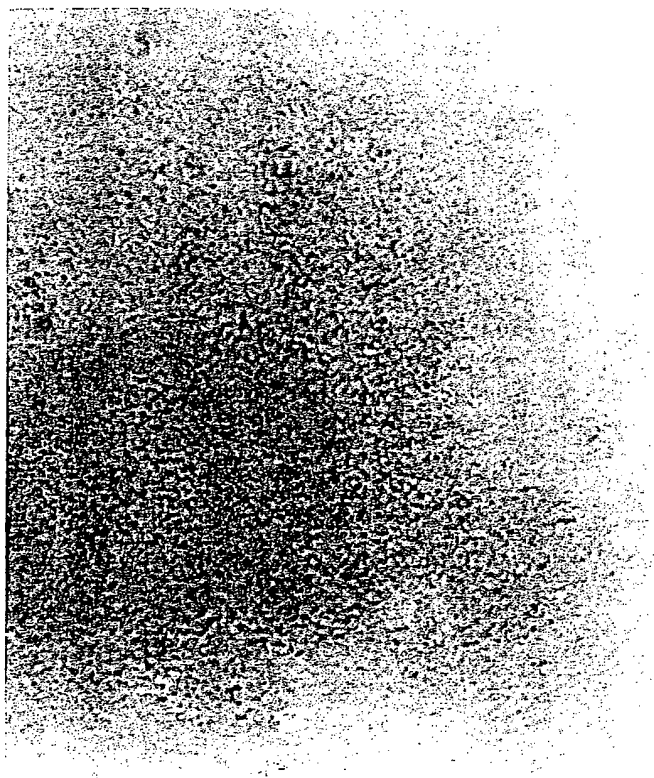


FIG.11B

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09041492.082901

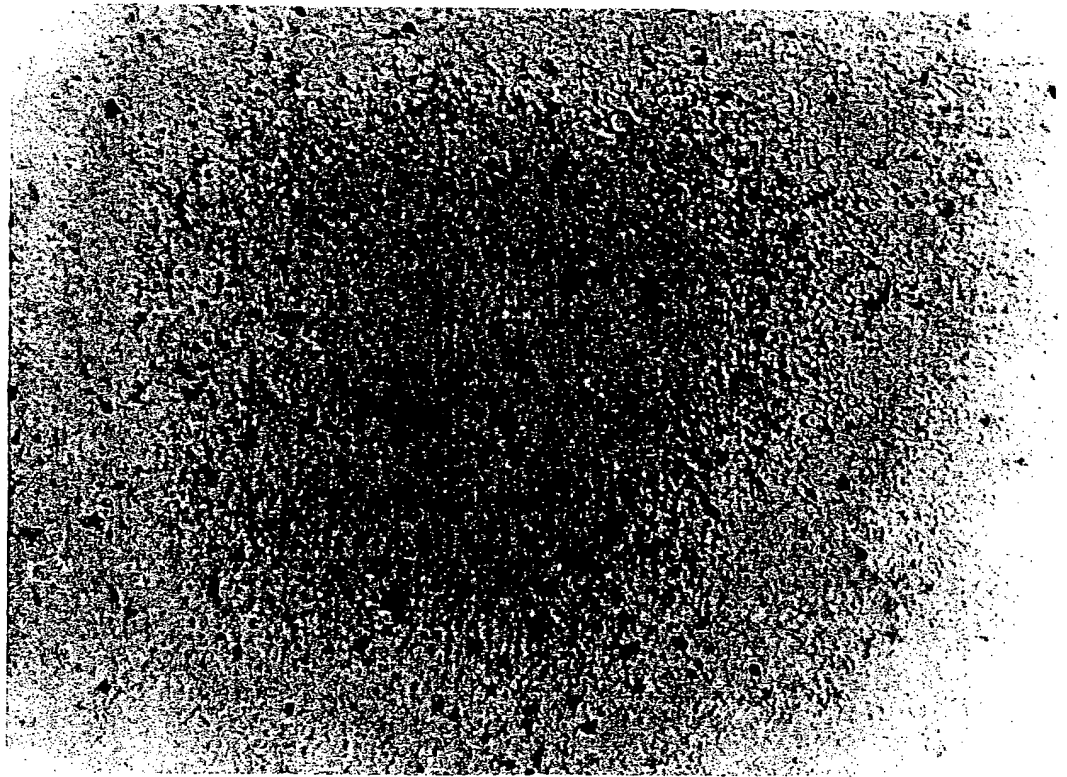


FIG.11C

094149 082901
105280 2647650

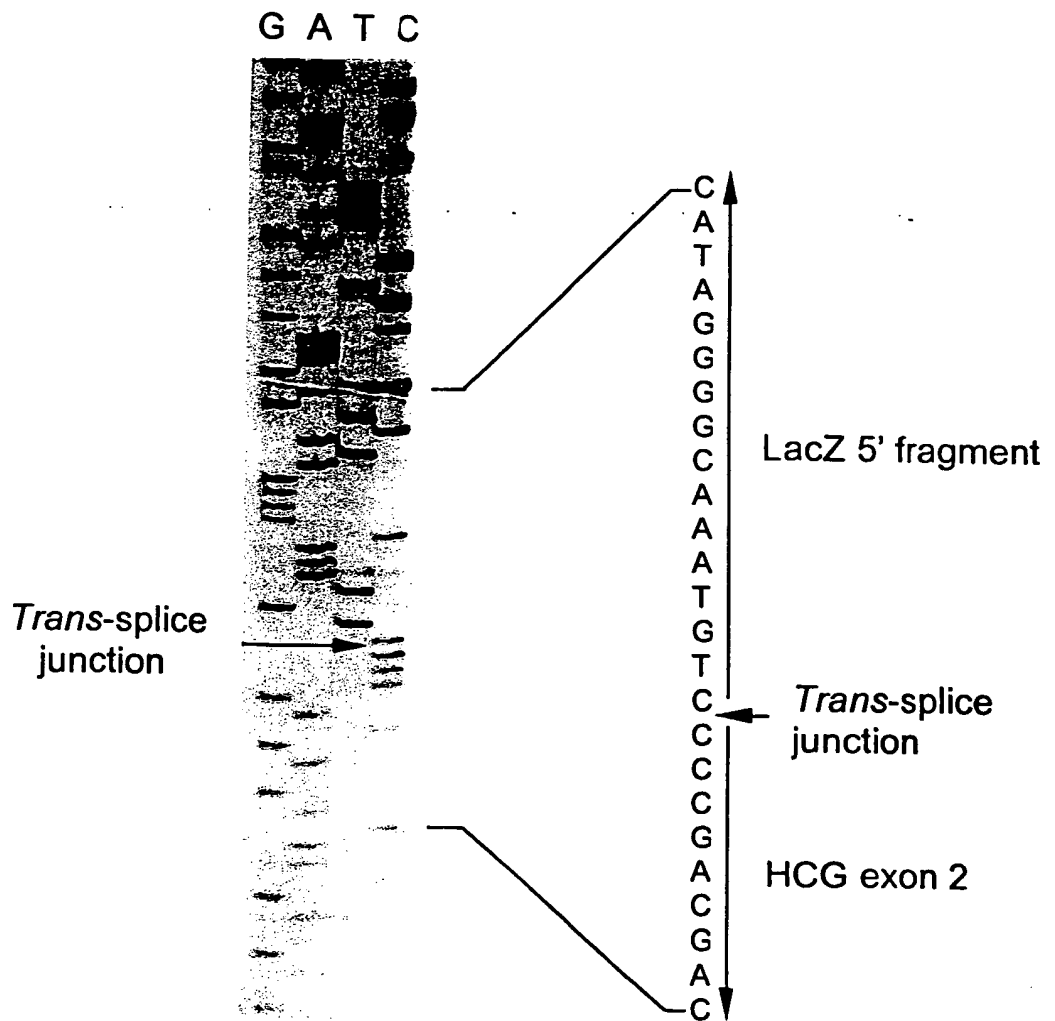
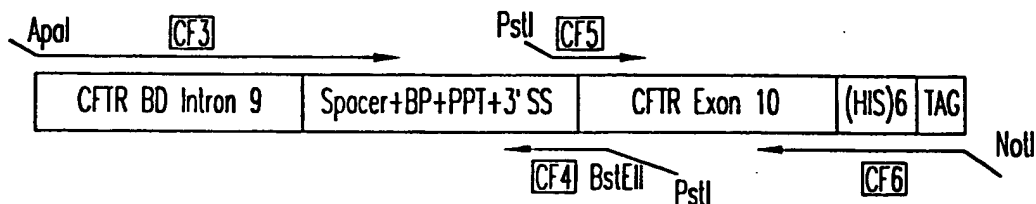


FIG.12A

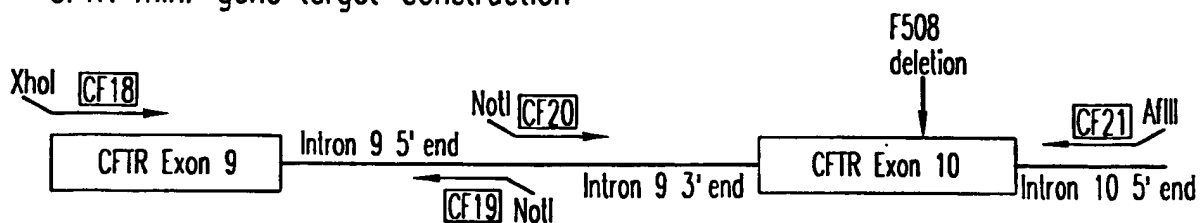
1. NUCLEOTIDE SEQUENCES OF THE *cis*-SPICED PRODUCT (285 bp):
 BioLac-TR1
GGCTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCCACGGATGGGTACAGTCTTG
 GCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCGTTTACAG/GCGGCTTCGCTAATAATG
 GGACTGGGTGGATCAGTCGCTGATTAAATATGATGAACGCGCAACCGTGGTCGGCTTACGGCGGTGATTT
 TGGCGATACGCGGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCGACCGCACCGCATCCAG
 Lac-TR2
 2. NUCLEOTIDE SEQUENCES OF THE *trans*-SPICED PRODUCT (195 bp)
 BioLac-TR1
GGCTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCCACGGATGGGTACAGTCTTG
 CGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCGTTTACAG/GCGCTGCTGCTGTGCTGCTGCT
 Splice junction
 HCR2
GAGCATGGGCGGACATGGGCATCCAAGGAGCCACTTCGGCCACGGTGCCG

FIG.12B

CFTR Pre-therapeutic molecule (PTM or "bullet")



CFTR mini-gene target-construction



Trans-splicing Repair

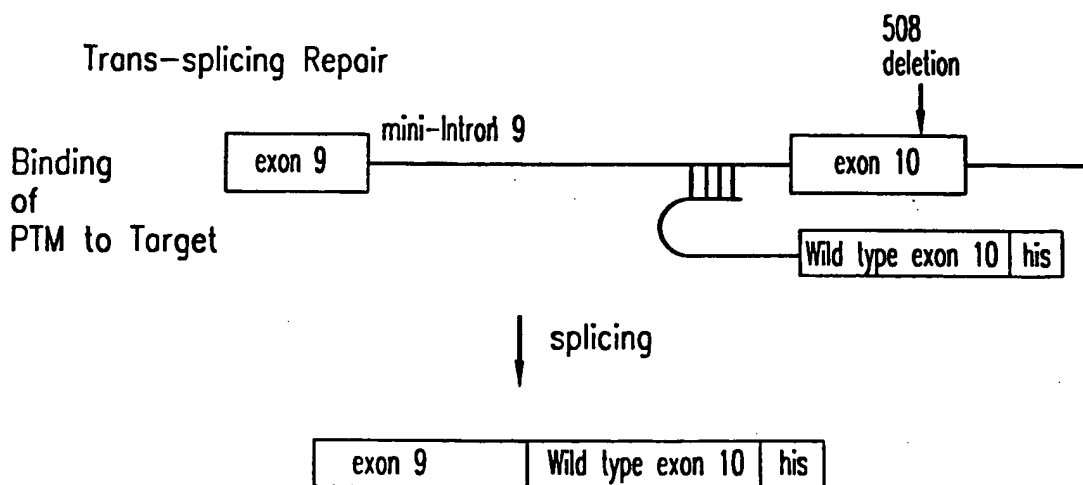


FIG.13

106280" 264T460

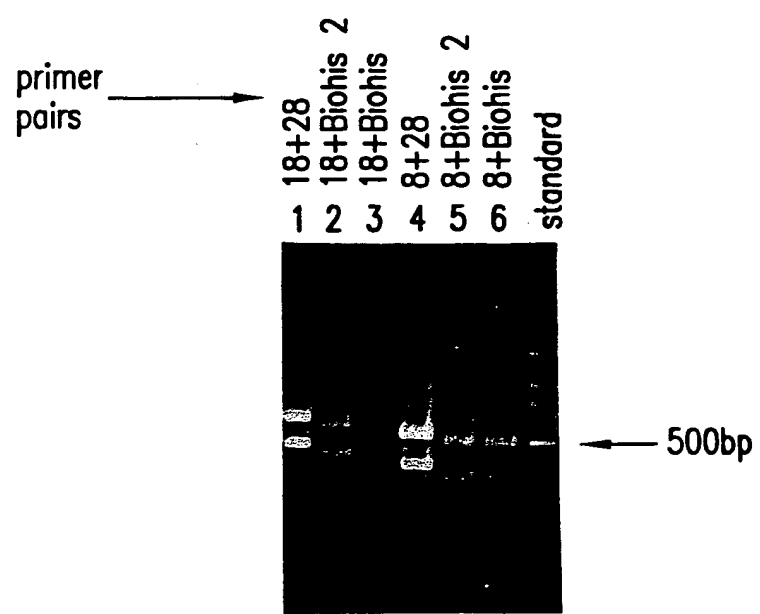
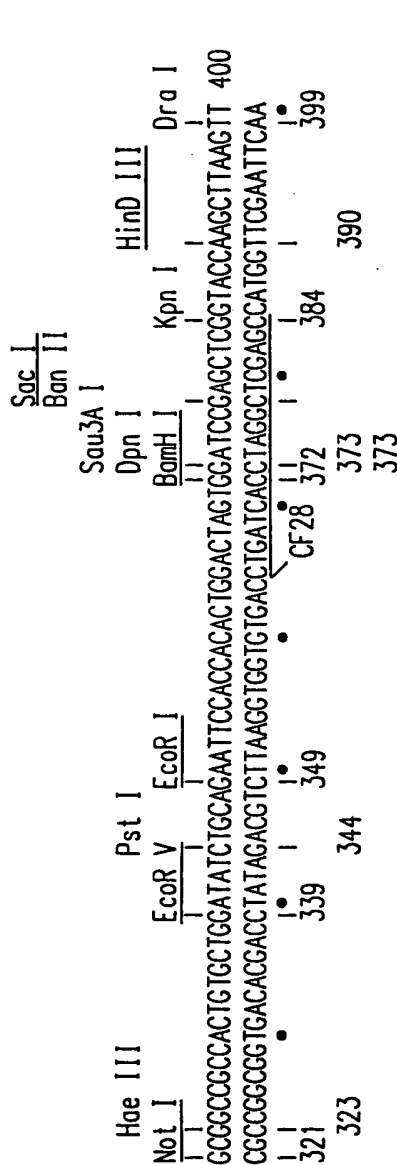


FIG.14



PRESENT IN PTM 3'UT
BUT NOT TARGET

TAAACCGCTGATCAGCCTCGACTGTCCTCTAGTTCAGCCAGCATCTGTTTCCCGCTCCCGCTCCCTTCCTTGACC 480
 ATTTGGCGGACTAGTCGGAGCTGACACCGAAGATCAACCGTCCGTAGACACAAACCGGGAGGGGCGGACGGAAGCACTGG

CTGGAAGGTGCCACTCCAC 500
 GACCTTCCACGGTGAGGGTG

Restriction Endonucleases site usage

Acc I	-	EcoR I	1	Nde I	-	Sau96 I	2
Apa I	1	EcoR V	1	Nhe I	1	Sca I	1
Apal I	-	Hae II	-	Not I	1	Sma I	-
Avr II	-	Hae III	2	PfIM I	-	Sph I	1
BamH I	1	HinC II	-	Pst I	2	SpI I	-
Ban II	2	HinD III	1	Pvu I	-	Ssp I	-
Bbe I	-	Hinf I	-	Pvu II	-	Stu I	-

FIG.15B

PTM [CFTR BD Intron 9] [Spacer+BP+PPT+3' SS] [CFTR exons 10-24] [(His) 6] TAG

+

F508 deletion

CFTR Target
(mini-gene)

CFTR Exons 1-9

CFTR Exons 10-24

Mini-intron 9
(~0.6kb)

Cotransfect PTM and target molecules in HEK 293 cells
and detect repaired CFTR mRNA by RT-PCR.

Repaired
CFTR mRNA

CFTR Exons 1-9 Exons 10-24 CFTR [(His) 6] TAG

FIG.16

20250114 09:01:26

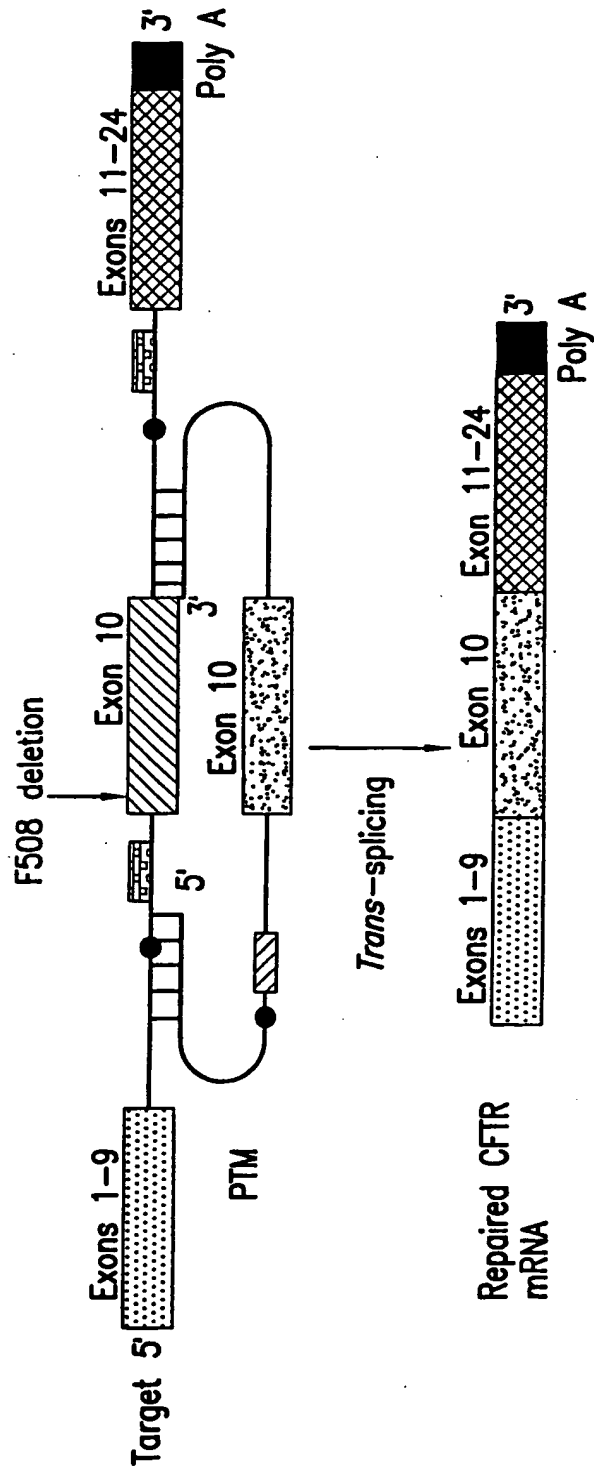
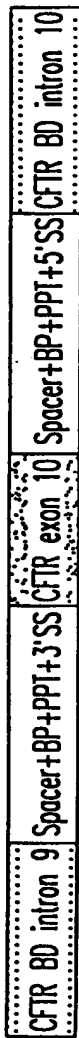


FIG.17

Double Trans-splicing Specific Target

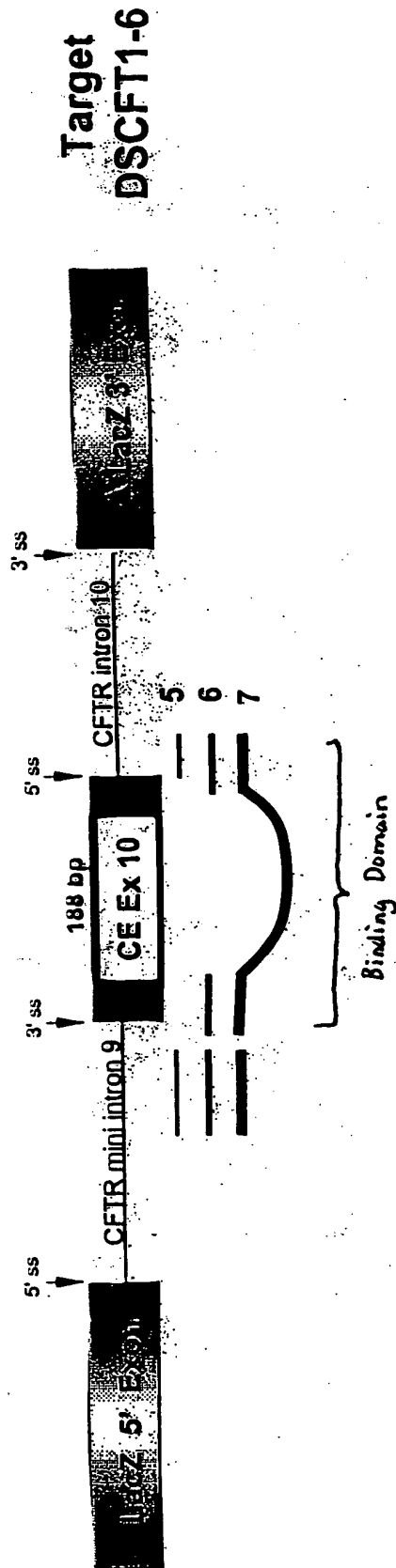


Figure 18

Double Splicing PTMs

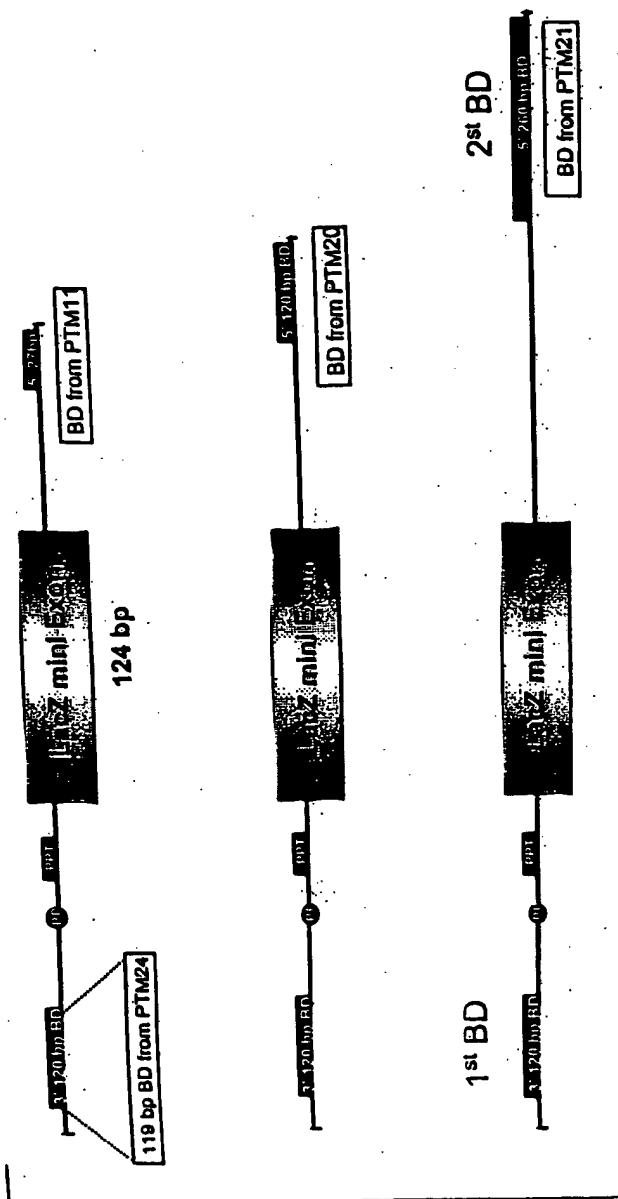


Figure 19

Double Trans-splicing β -Gal Model

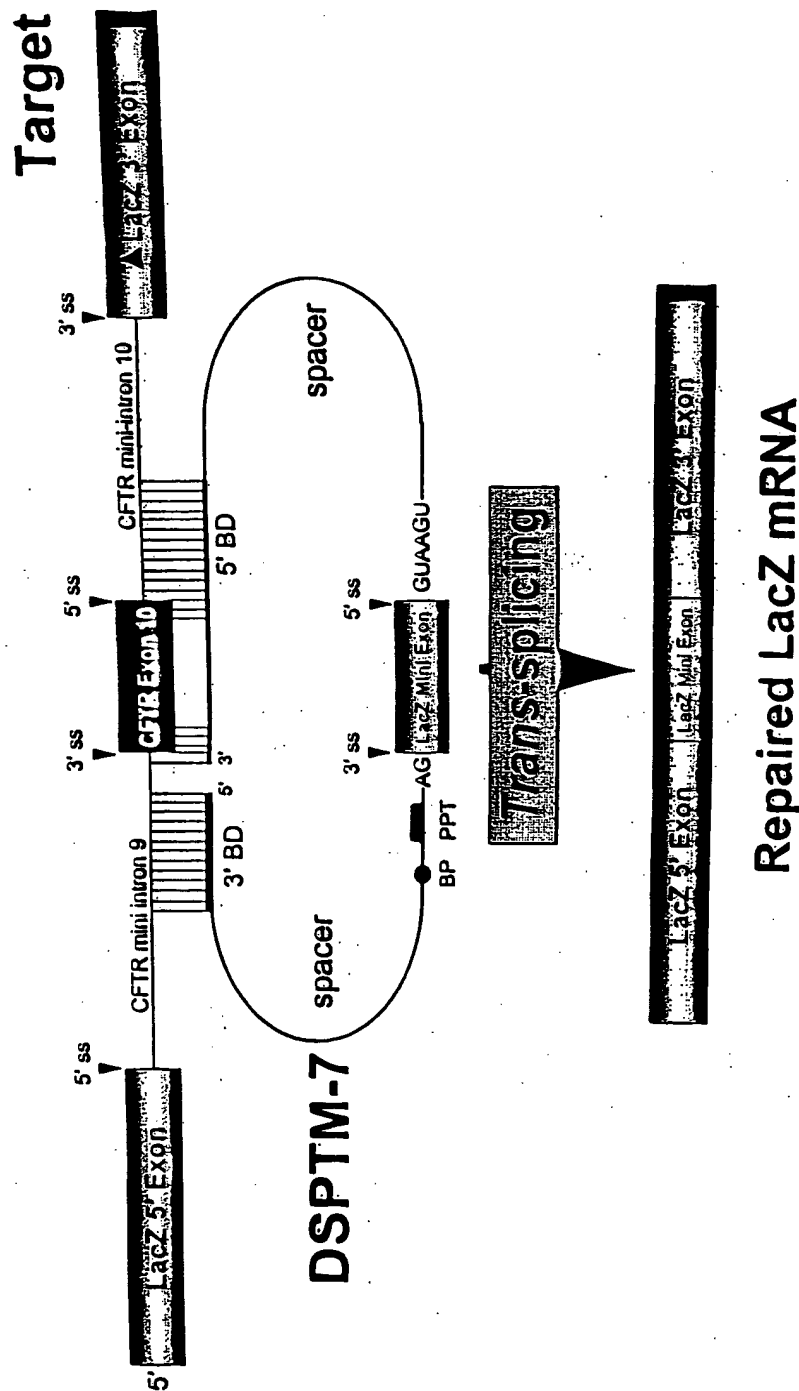
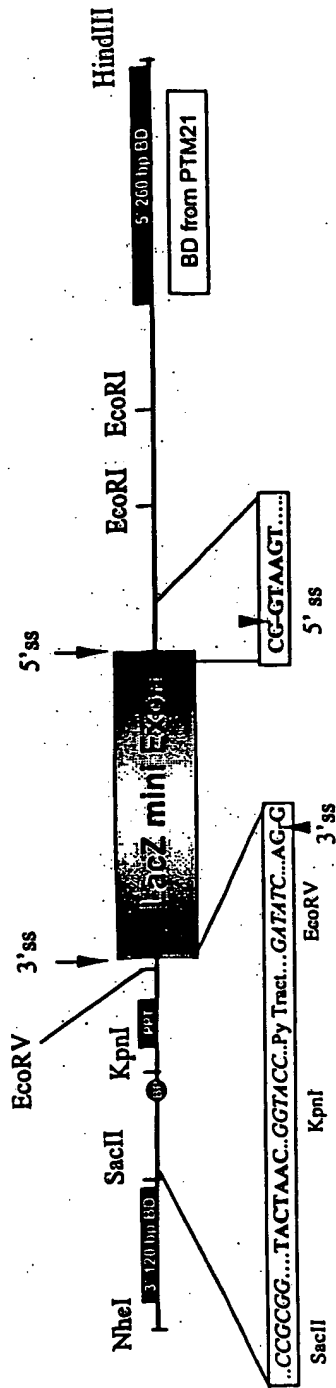


Figure 20

Important Structural Elements of DSPTM-7: (Double splicing PTM with all the necessary splice elements i.e. has both 3' and 5' functional splice sites and the binding domains)



(1) 3' BD (120 BP) : GATTCACCTTGCTCCAAATTATCATCCTAAGCAGAAGTGATATCTTATTGTAAAGATTCTATTAACTCATTGATTC
AAAATATTAAATACTTCCTGTTTCATACTCTGCTATGCAC

(2) Spacer sequences (24 bp): AACATTATTATAACGTTGCTCGAA

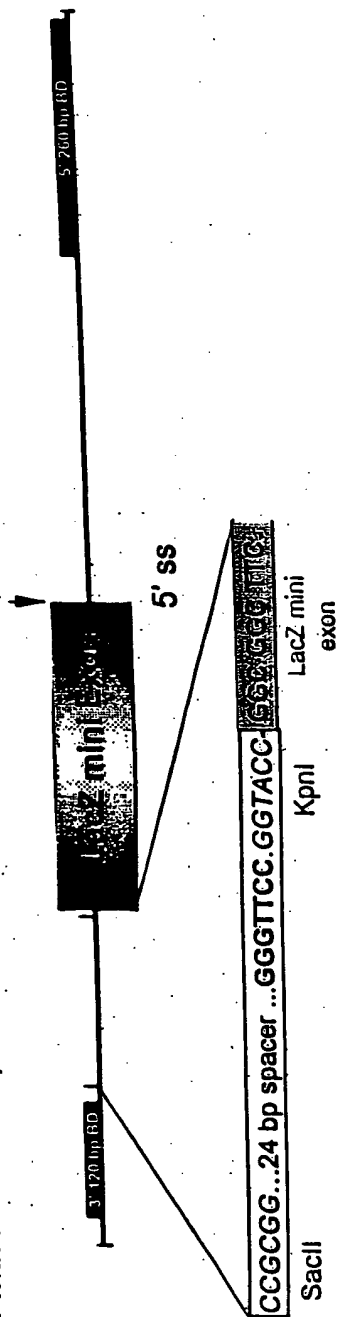
(3) Branch point, pyrimidine tract and acceptor splice site: TACTAAC T GGTAAC TCTTCTTTTTTTTTT GATATC CTGCAG **GGG GGG**
3' ss LacZ mini exon
EcoRV PPT KpnI BP

(4) 5' donor site and 2nd spacer sequence: **CTAAGG** GTTAAGT GTTATCACCAGATATGTGCTAACCTGATTCGGGCCCTTCGATACG
5' ss LacZ mini exon
CTAAGATCCACCGG

(5) 5' BD (260 BP) : TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTTGAAATTCATGCTTTGATGACGCTTCTGTATCTATATTCATTCATTGGAA
ACACCAATGATTTTCTTTAATGGTGCTGGCCTGGCATAATCCTGGAAAACCTGATAACACAAATGAAATCTTCCACTGTGCTTAA
AAAAACCCCTCTGAAATTCCTCCATTCTCCCATATCATCATTAACCTGAACCTCTGGAAATAAAACCCATCATTTAACTCA
TTATCAAAATCACGC

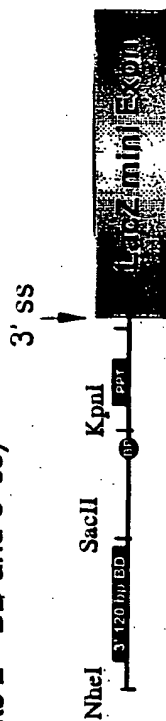
Figure 21

DSPTM8 : (▲ 3' ss: 3' splice elements i.e. BP, PPT & AG dinucleotide has been deleted and replaced with random sequences, but still has the functional 5' splice site)



Mutants

PTM29 (lacks 2nd BD and 5' ss)



PTM30 (lacks 1st BD and 3' ss)



Figure 22

Accuracy of Double Trans-splicing Reaction

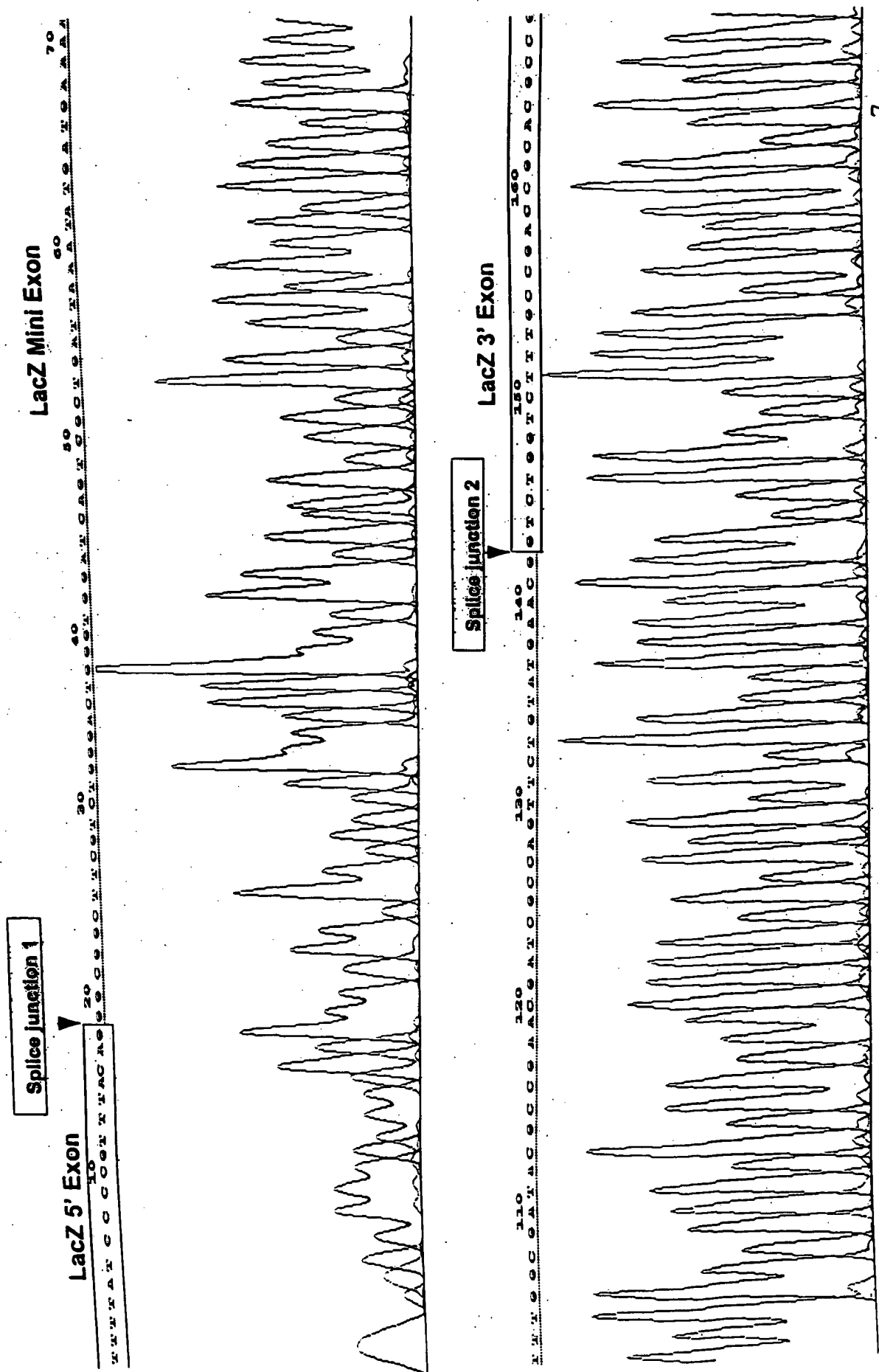


Figure 23

Double Trans-splicing Produces Full-length Protein



β-gal →
(120 kDa)

1 2 3 4 5 6 7

Lane 1: DSCFT1.6 Target alone 25 μg
 Lane 2: DSPTM7 25 μg
 Lane 3 Target + PTM #6 25 μg
 Lane 4: Target + PTM #9 25 μg
 Lane 5: Delta 3' splice mutant alone 25 μg
 Lane 6: Target + Delta 3' ss 25 μg
 Lane 7: Target+PTM29+30 (mutants) 25 μg

Figure 24

Restoration of β -Gal Function by Double Trans-splicing

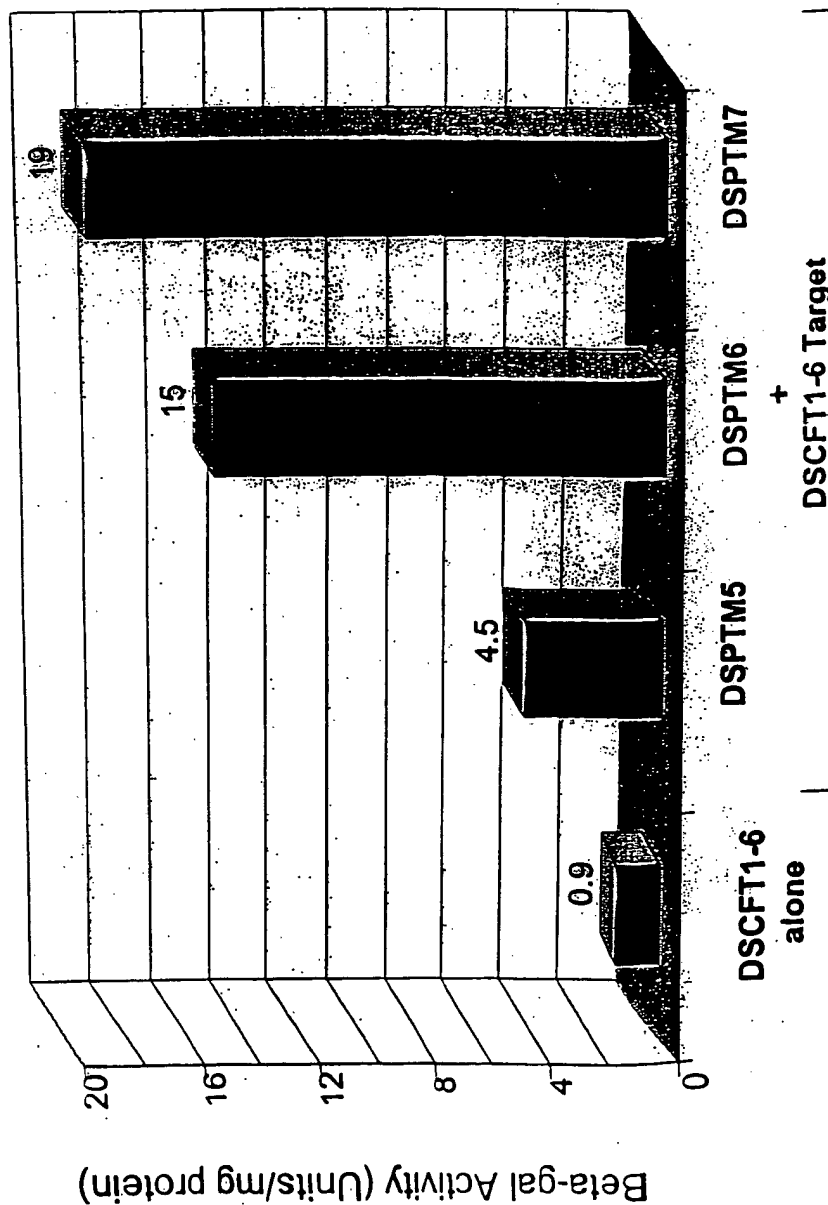


Figure 25

Restoration of β -gal activity is due to double RNA trans-splicing events

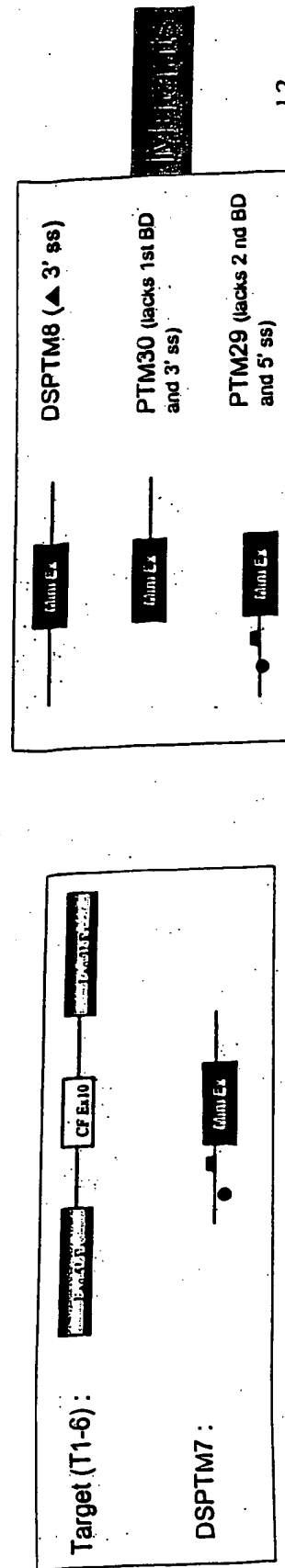
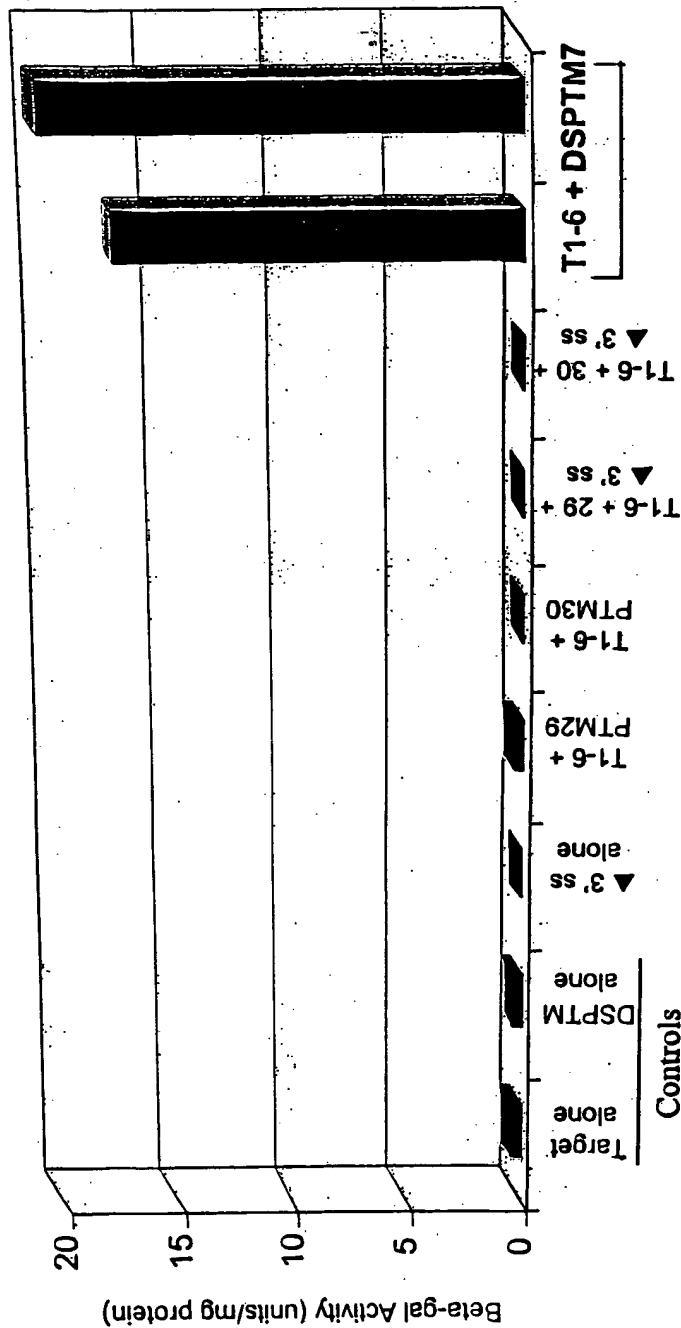


Figure 26

Double Trans-splicing: Titration of Target & PTM

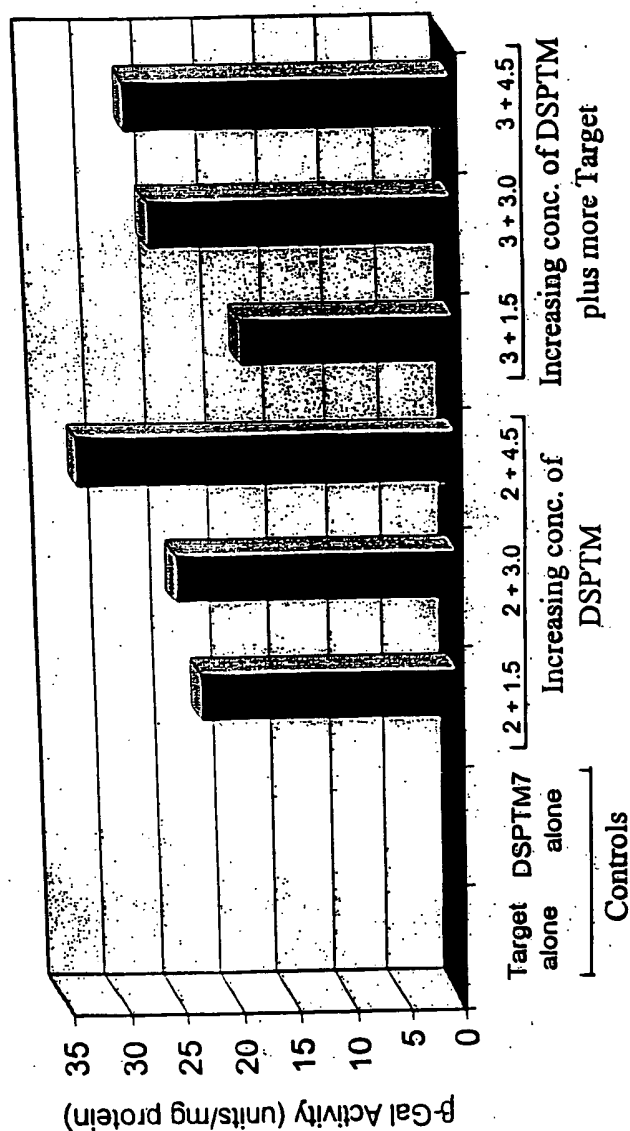
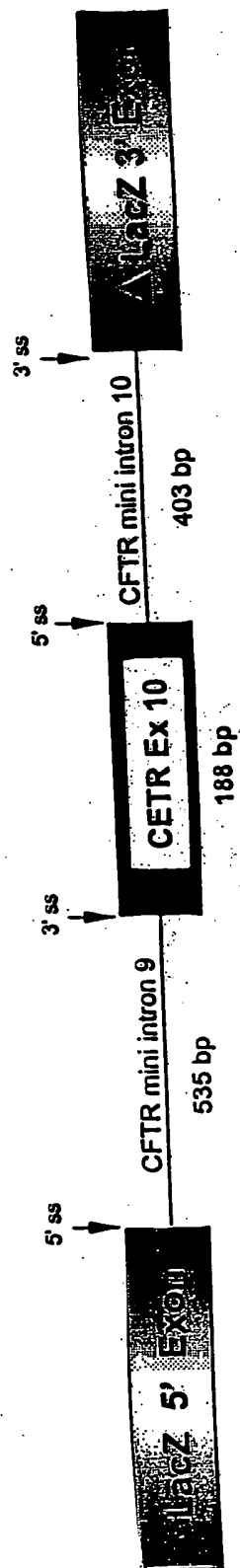


Figure 27

DSCFT1-6 (Specific Target):



DSHCGT1 (Non-specific Target):

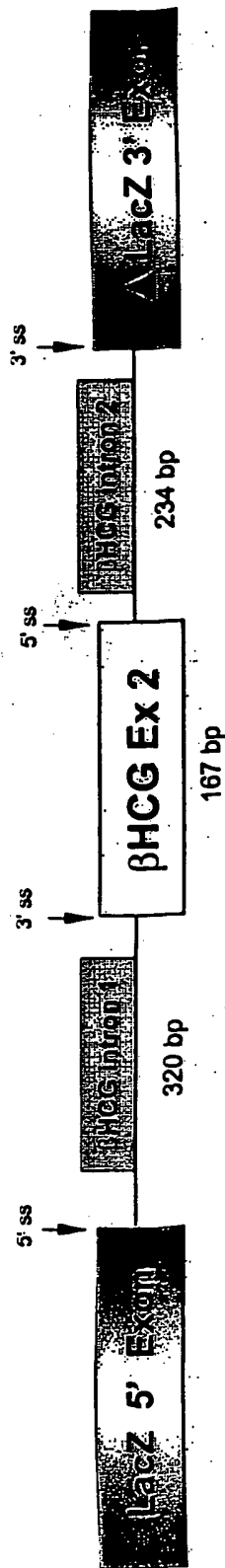


Figure 28

106280-264450

Specificity of double *trans*-splicing Reaction

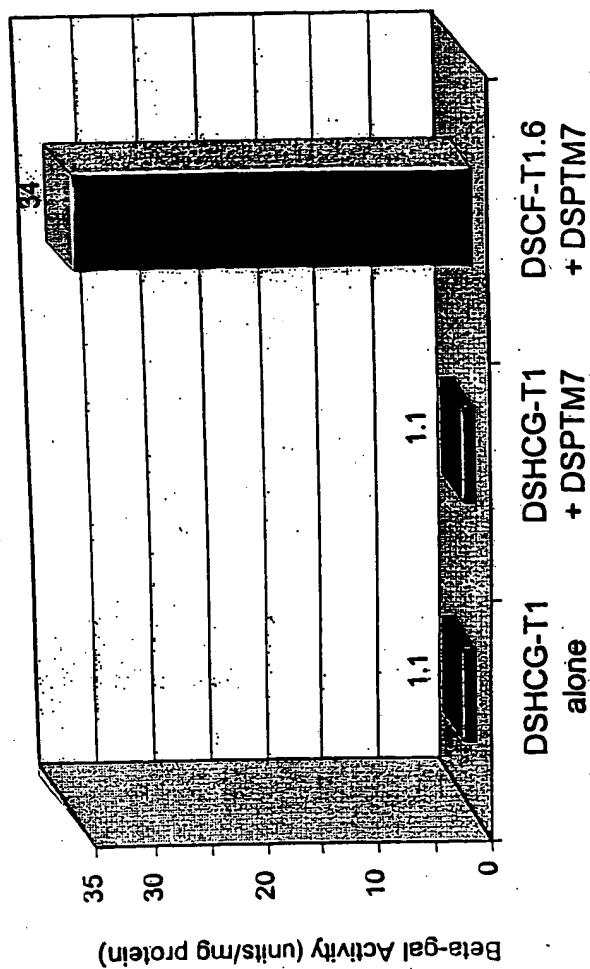


Figure 29

Repaired full length CFTR mRNA

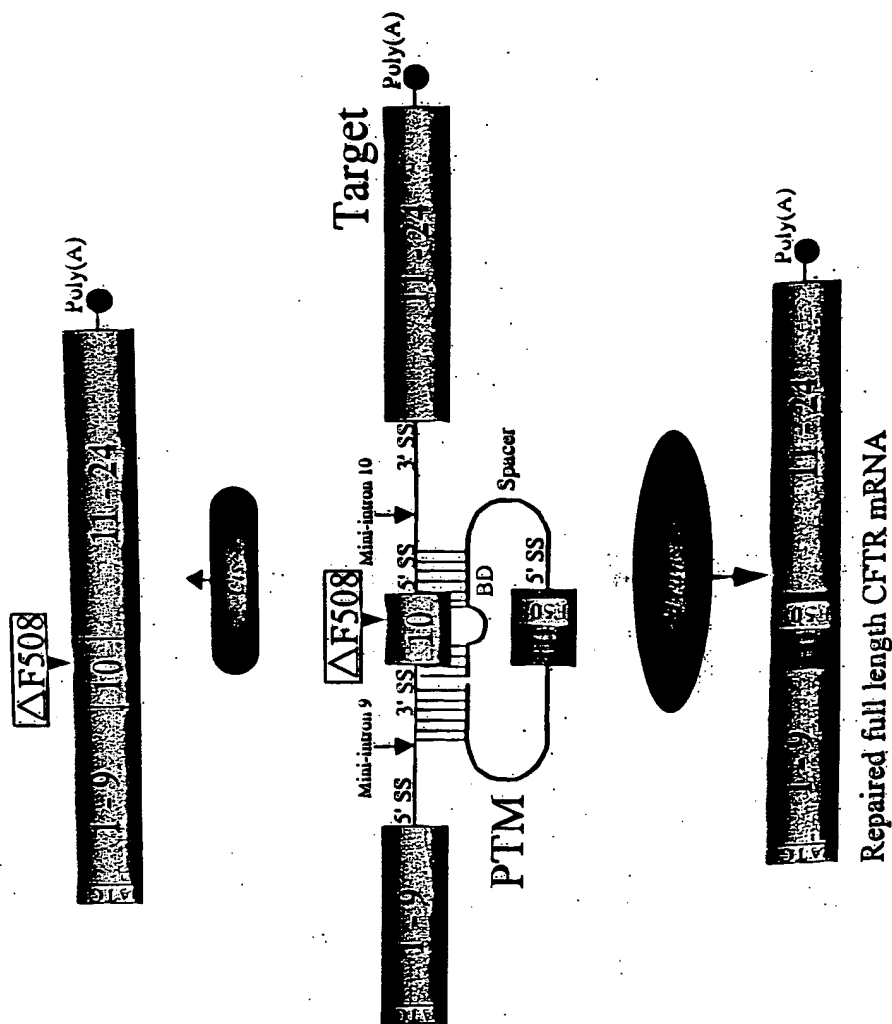
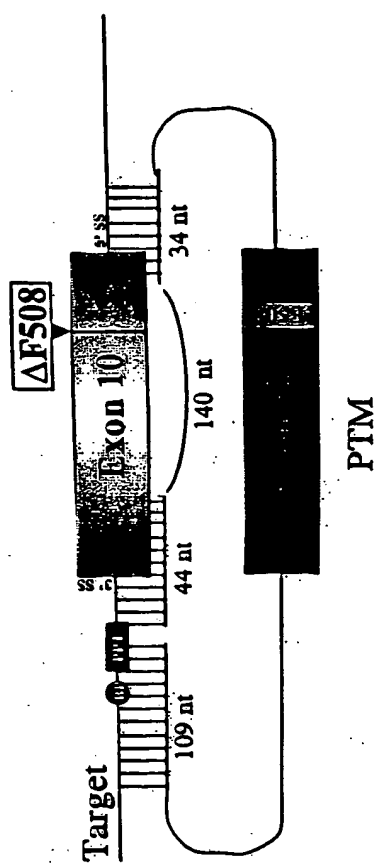


Figure 30

INTRONIN

PTM with a long binding domain masking two splice sites and part of exon 10 in a mini-gene target.



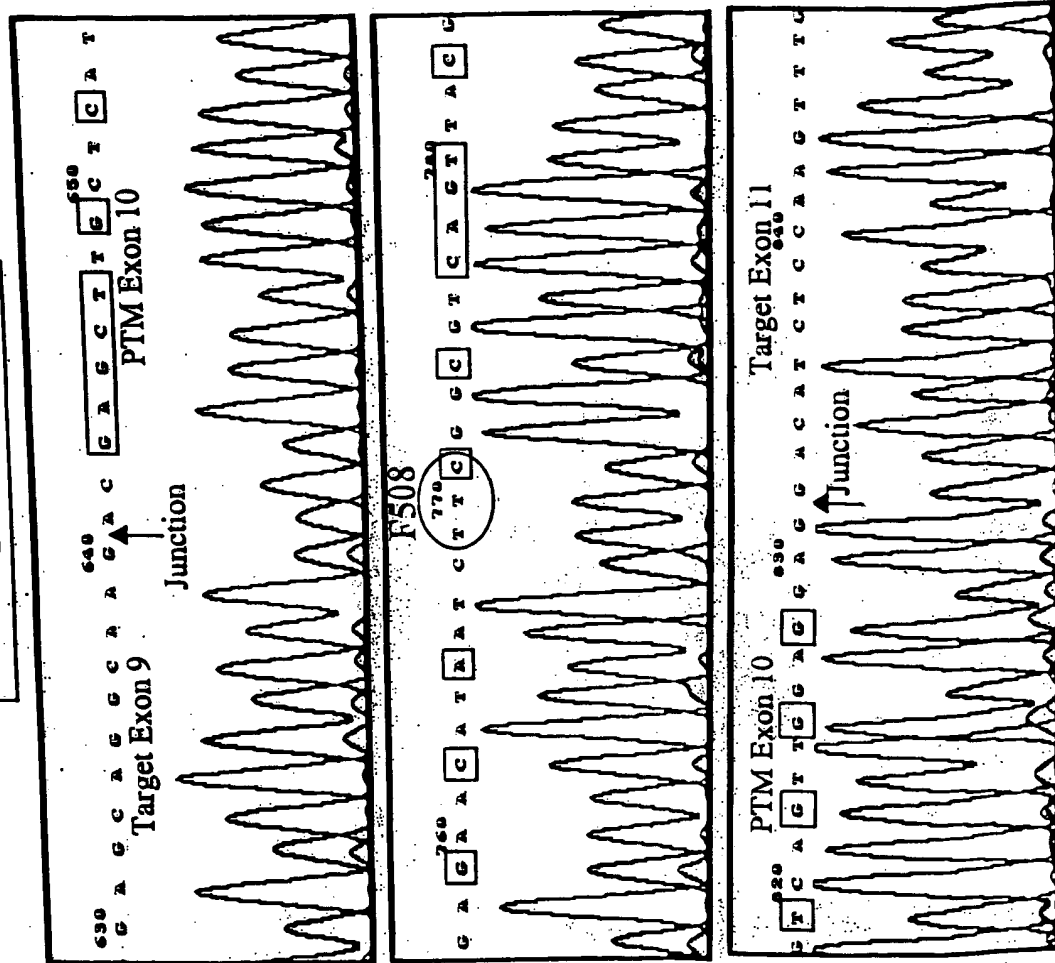
ACGAGCTTGCTCATGATCATGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAAATTCCTCGG
 GCCGCATCAGCTTTTCAGCCCAATTTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAT
 CTTCGGCGTTCAGTTACGACGAGTACCGCTATCCGCTCGGTGATTAAGGCCCTGTCAGTTGGAGGAG

MCU in exon 10 of PTM
 88 of 192 (46%) bases in PTM exon 10 are not complementary to
 its binding domain (bold and underlined).

Figure 31

INTRONN

Sequence of a double *trans*-spliced product



MCU in PTM exon 10

Figure 32

—

68 2077

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CFTR Repair: 5' Exon Replacement

Schematic diagram of a PTM binding to the splice site of intron 10 of a mini-gene target

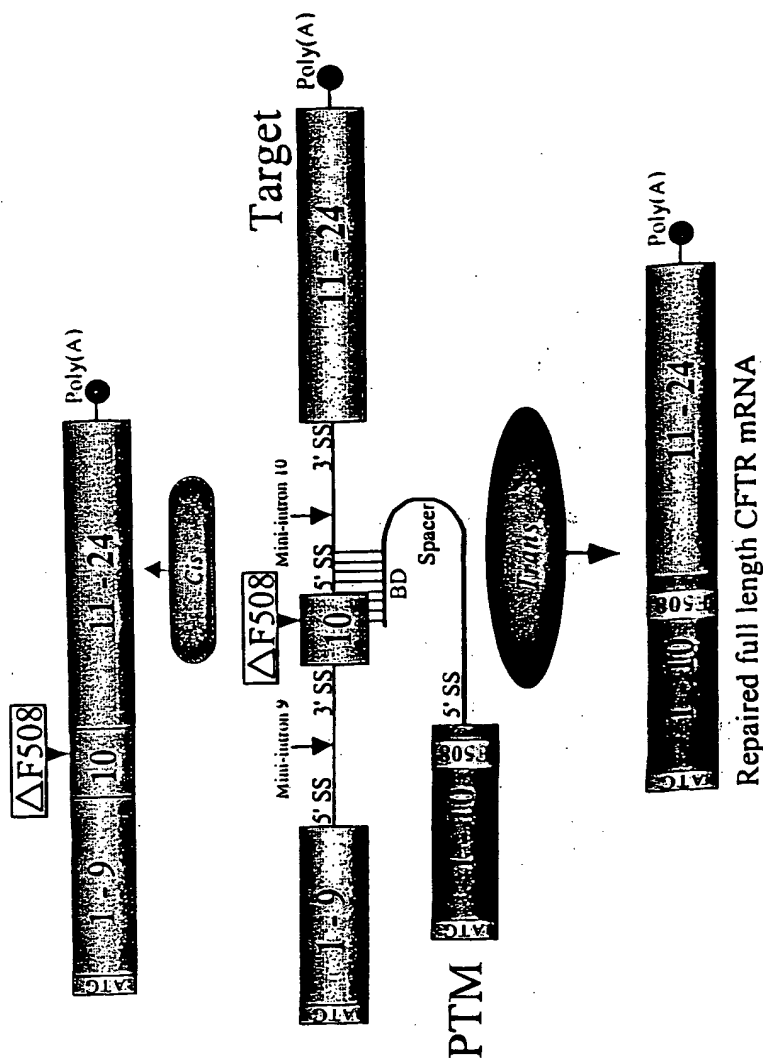
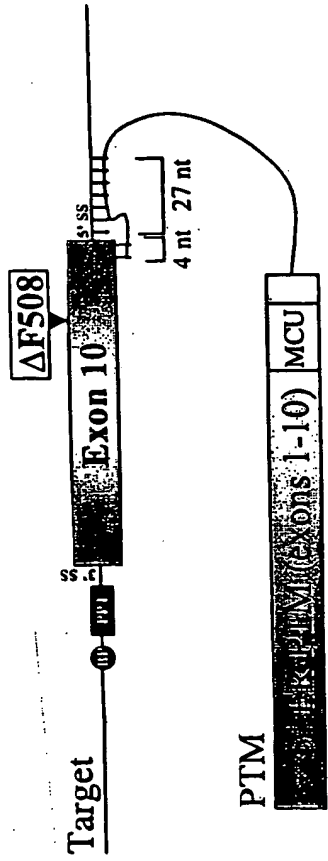
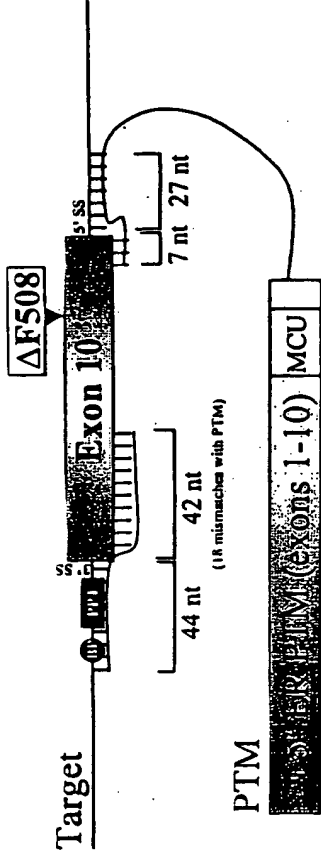


Figure 33

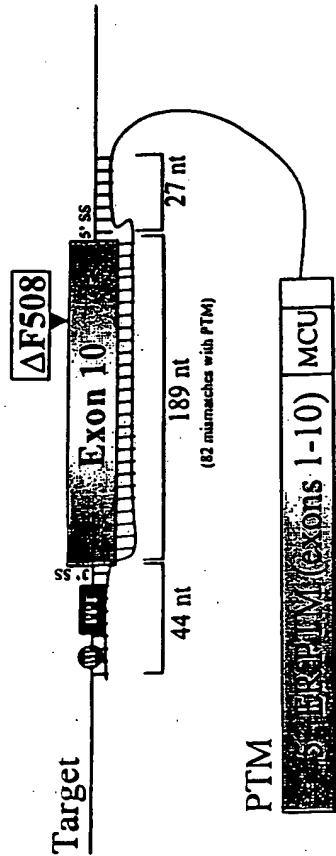
20250726T1550



PTM with a short binding domain masking a single splice site in a mini-gene target.



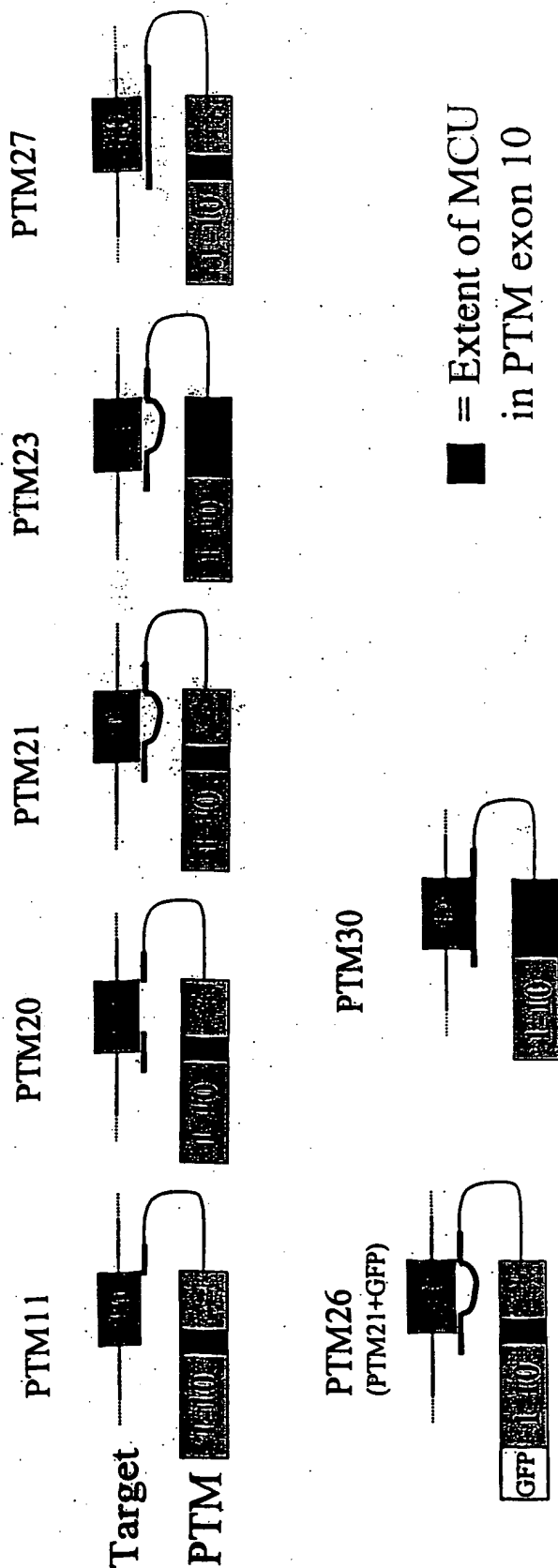
PTM with a long binding domain masking two splice sites in a mini-gene target.



PTM with a long binding domain masking two splice sites and the whole of exon 10 in a mini-gene target.

Figure 34

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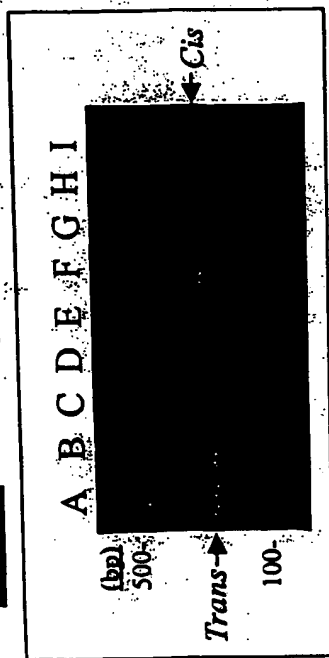


MCU in exon 10 of PTM
88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain.

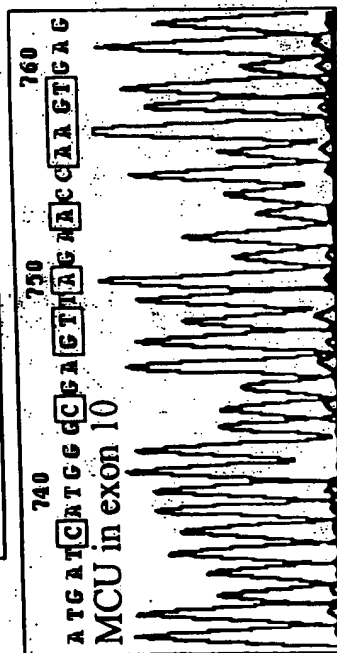
ACGAGCTTGCTCATGATGATCGGGGAGTTAGAACCAAGTGAAGGCAAGATCAACATTCGG
GCCGATCAGCTTTGCGAGCCAAATTCAGTTGGATGCGCGGTACCATCAAGGAGAACATAT
CTTCGGGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCCTGTCAGTTGGAGGAG

Figure 35

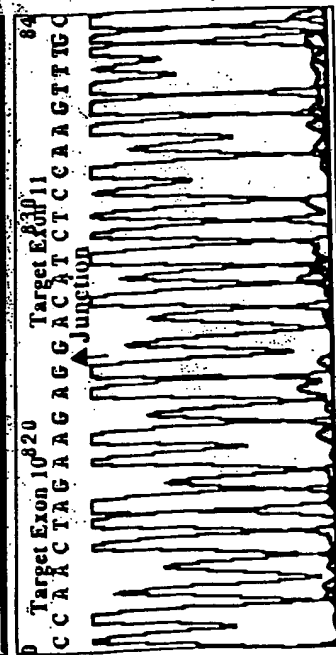
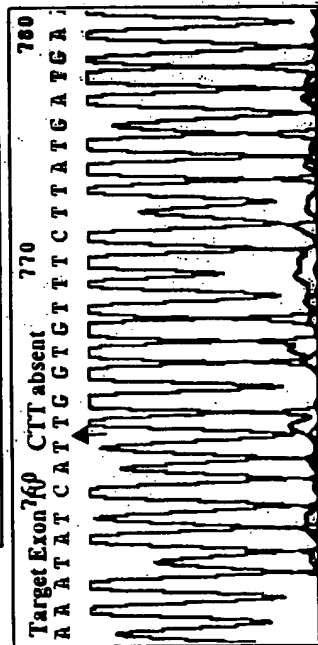
PrM Target



B.
Trans-spliced product
[Primers CF93 + CF111]



A.
Cis-spliced product
[Primers CF1 + CF111]



A

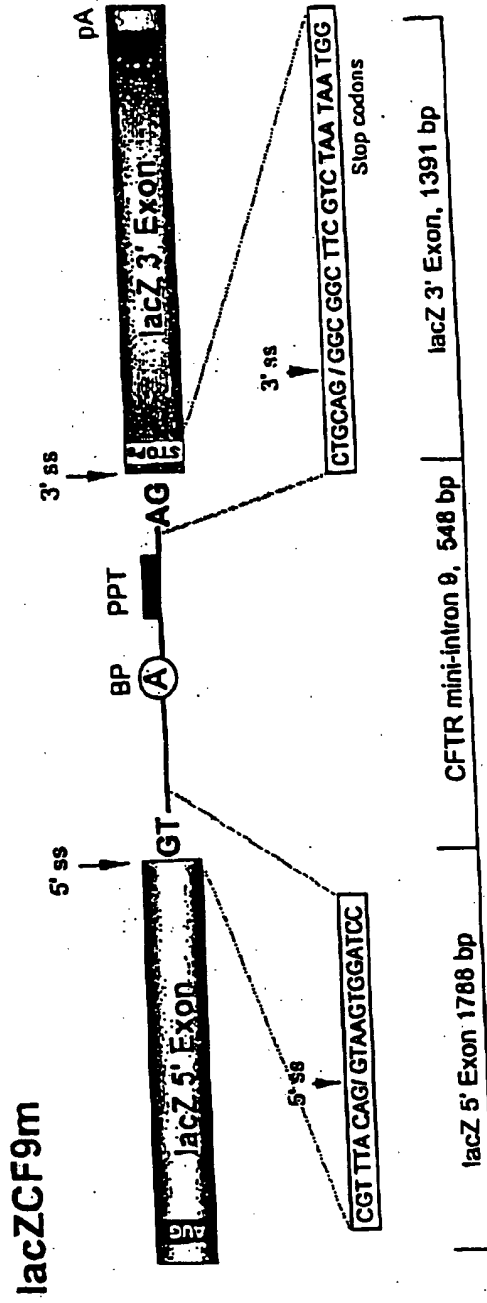
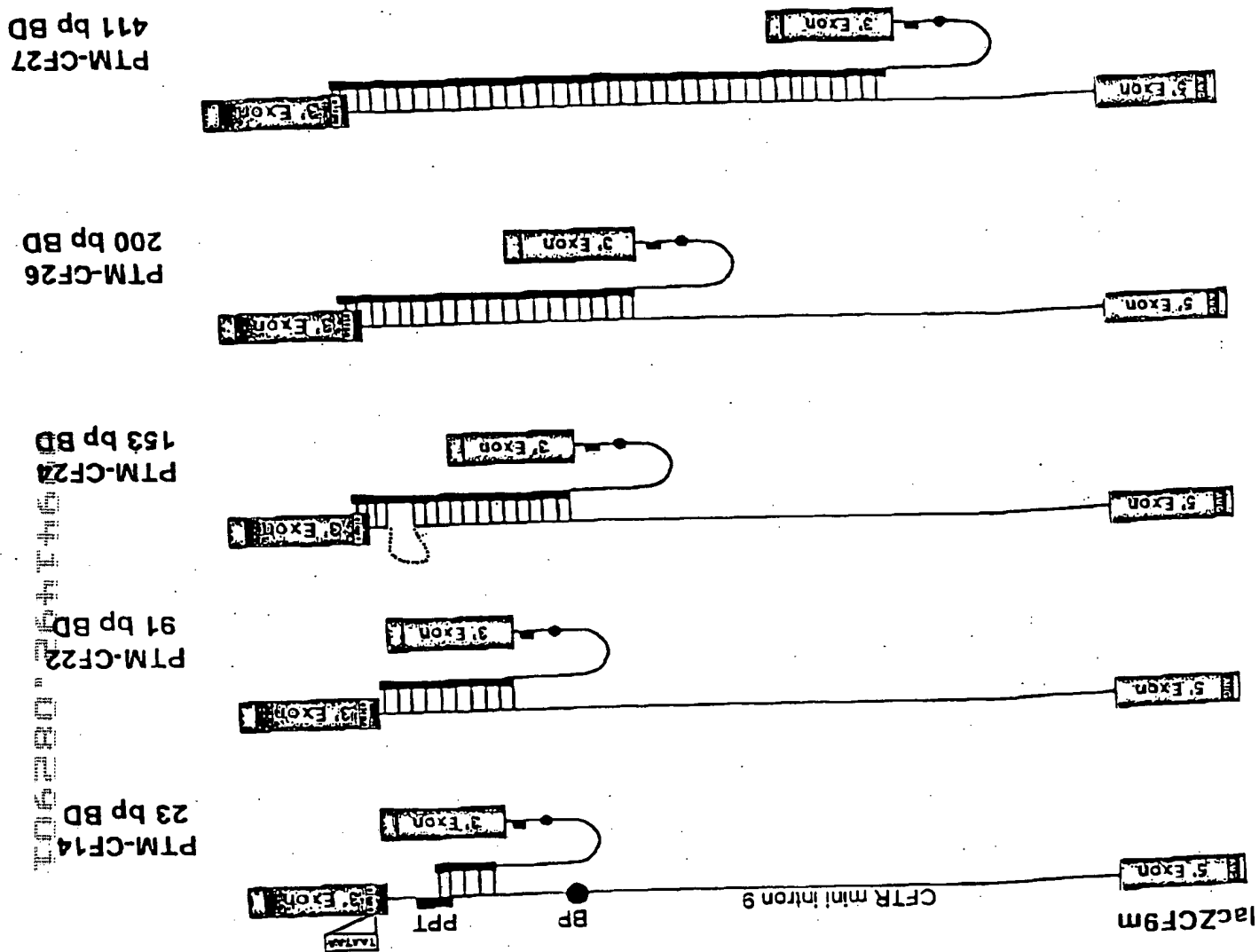
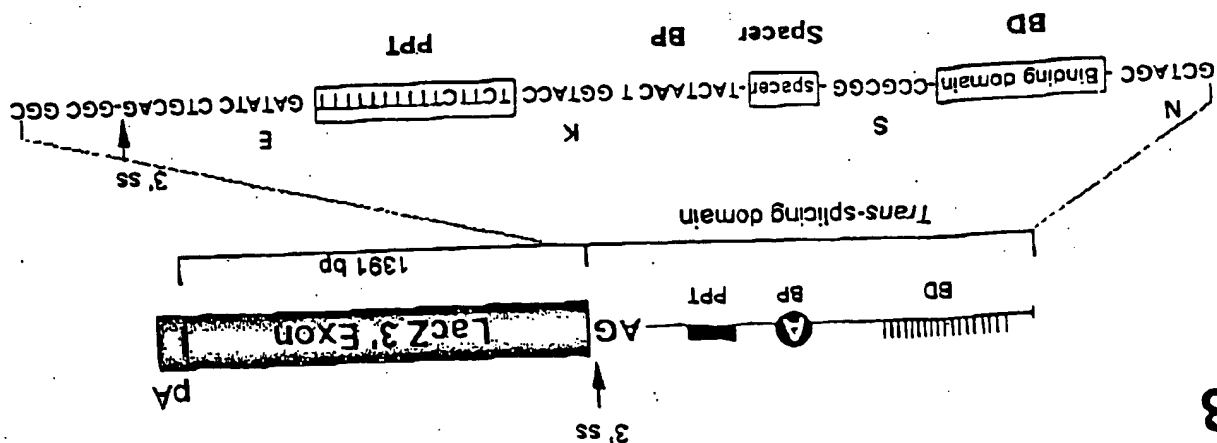


Figure 37 A

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68 78 87

B



C

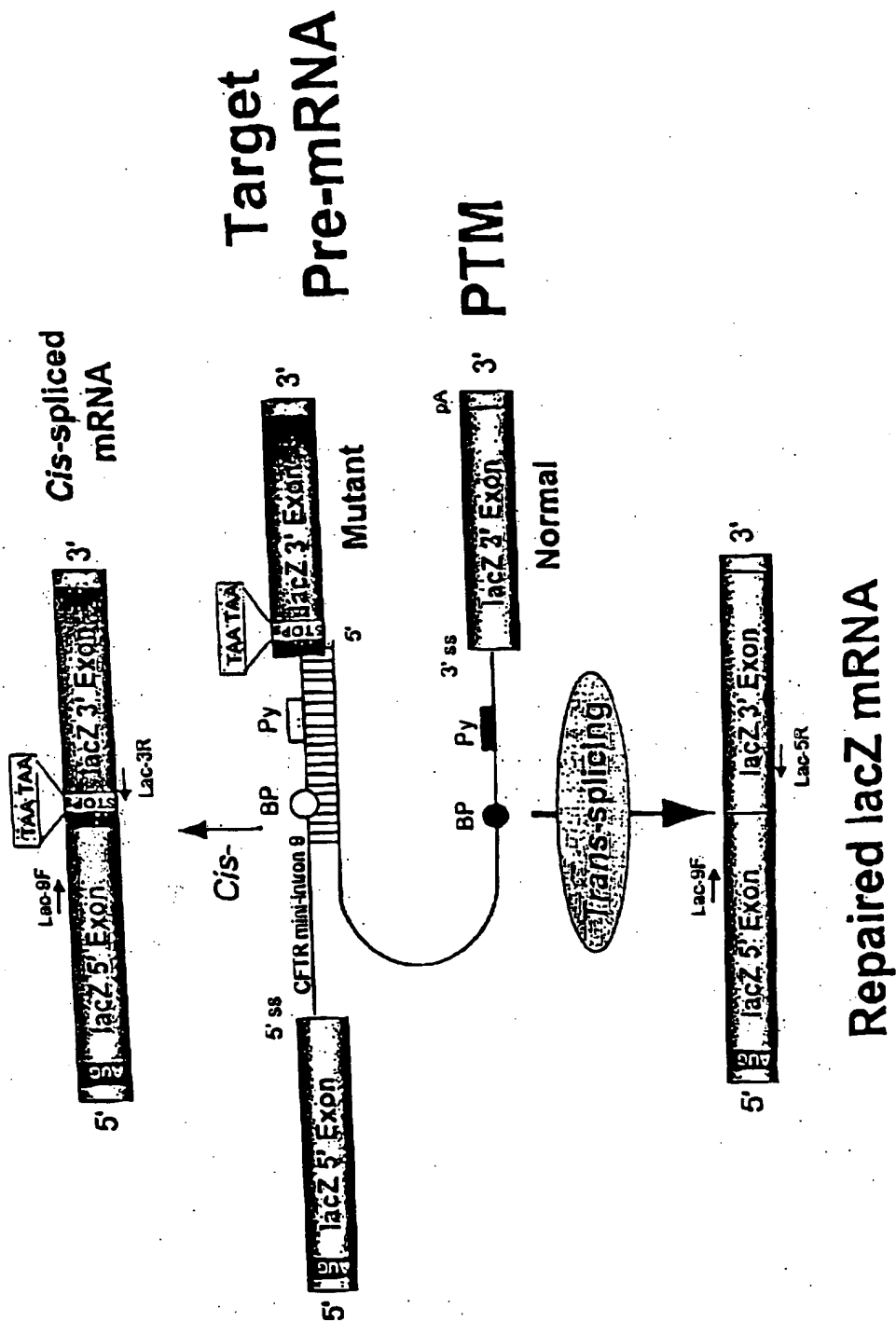


Figure 37C

A

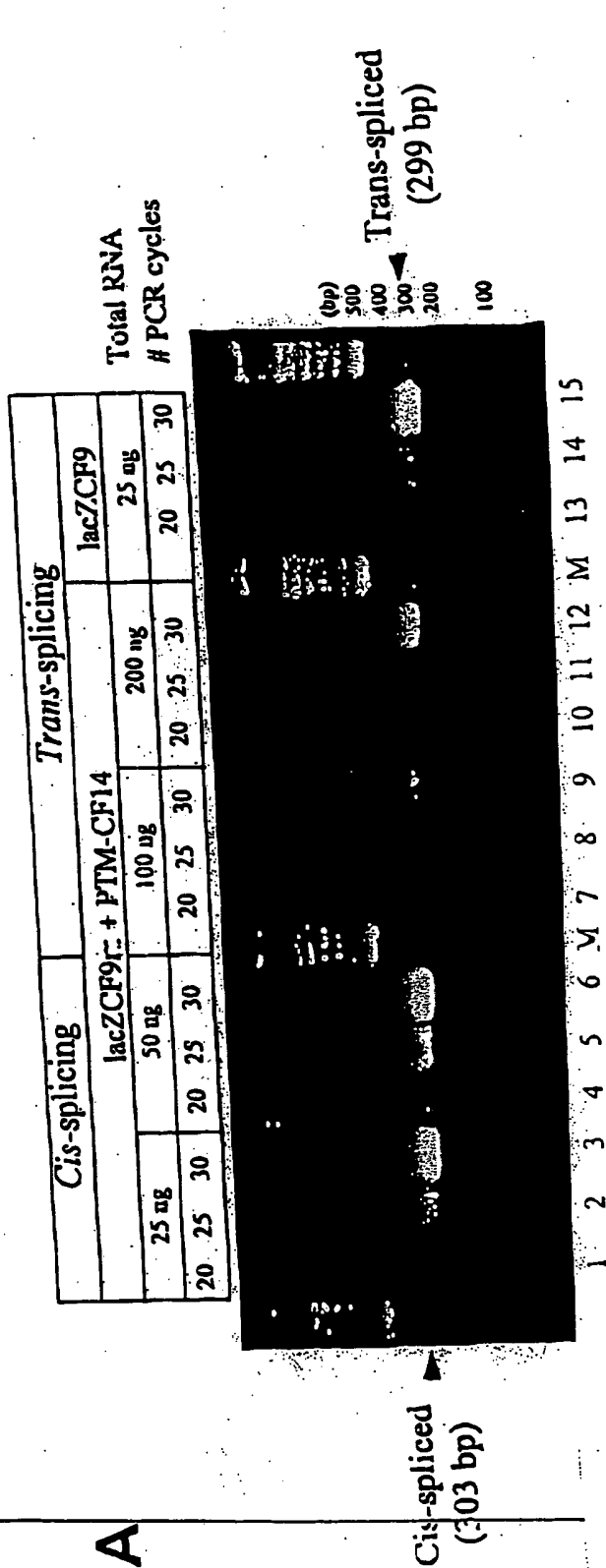
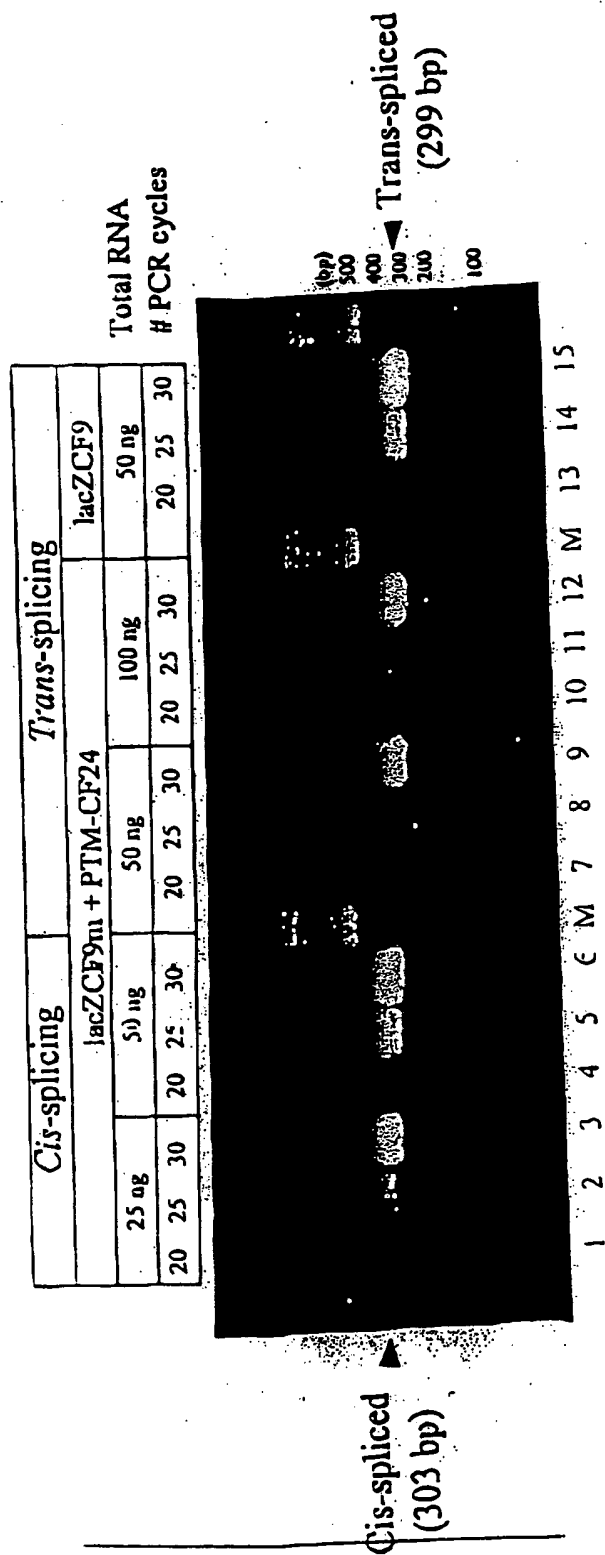


Figure 38A



106280" 2541560

B

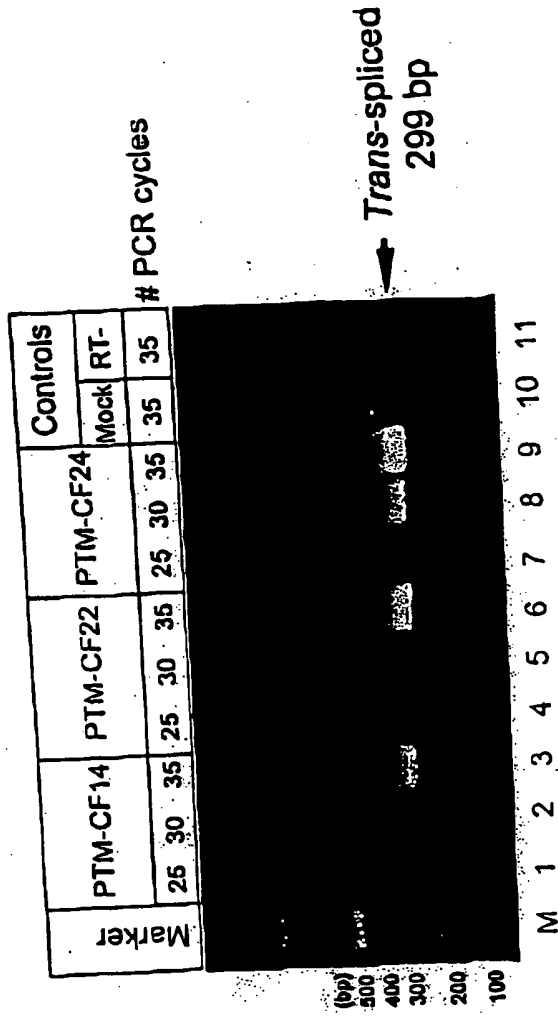


Figure 38B

106280" 26414650

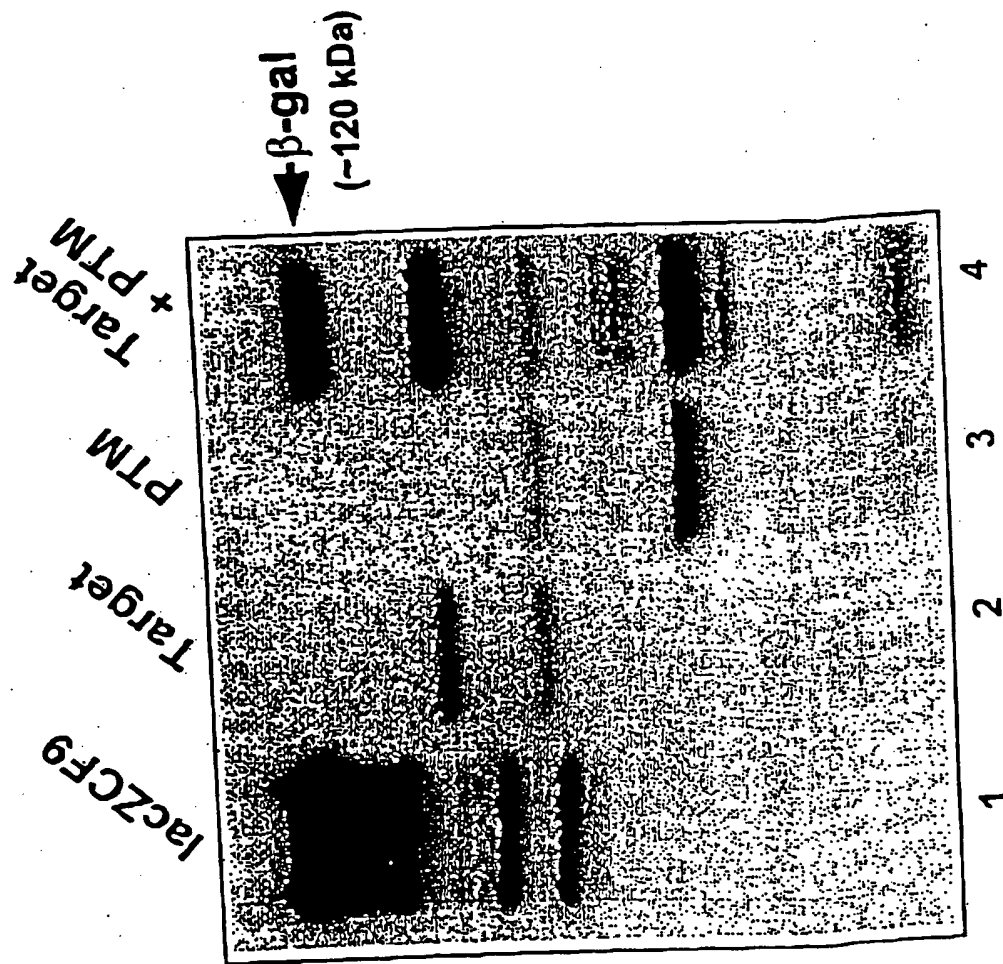


Figure 39

05941492-082901

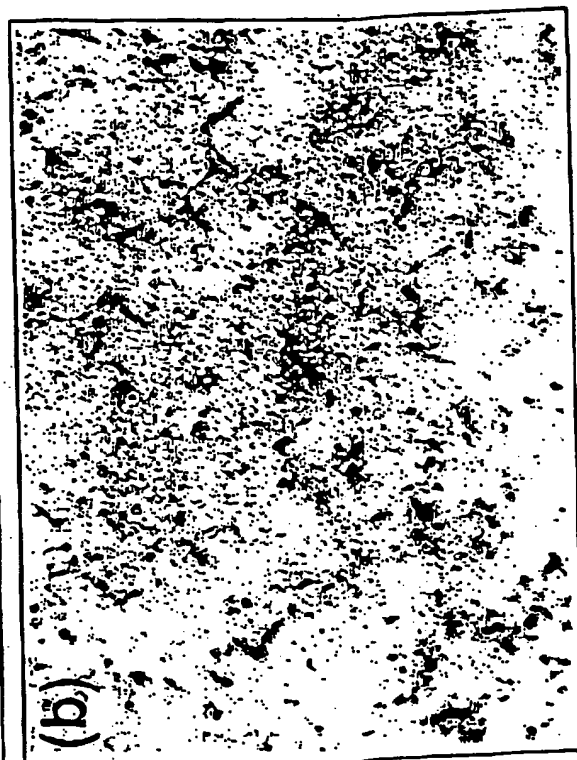
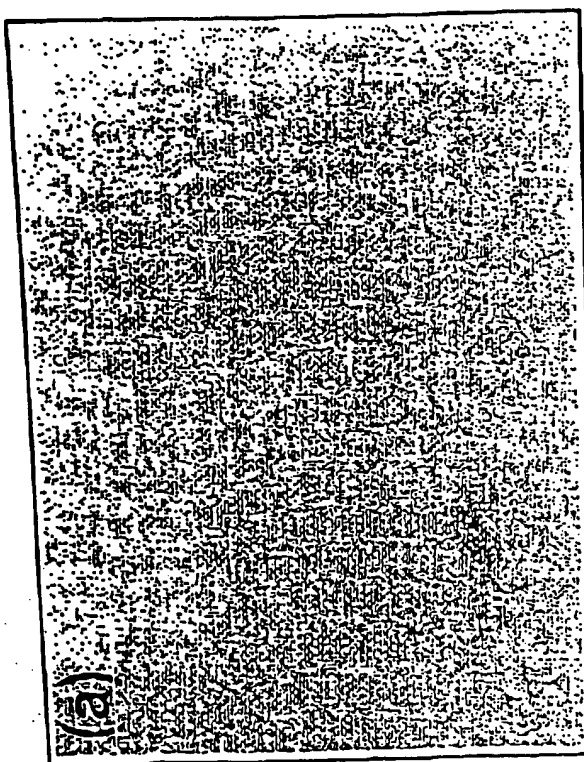


Figure 40A

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105280" 254T450

B

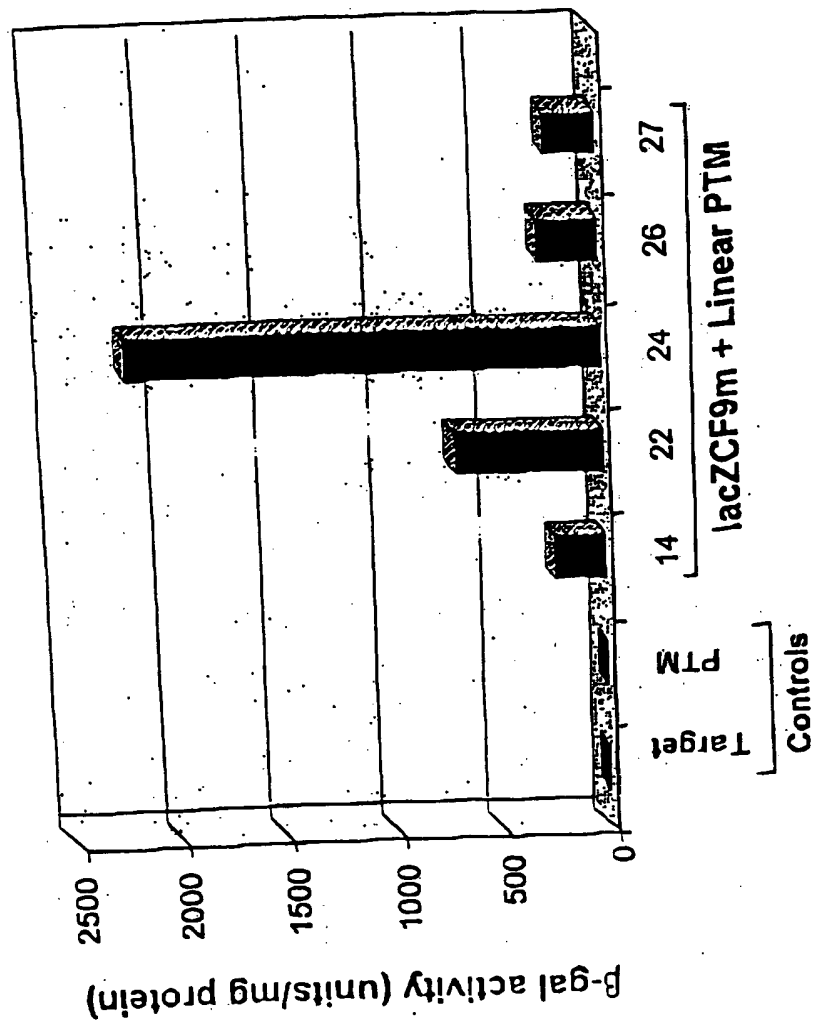


Figure 40B

54 of 89

FO6280" 2644650

C

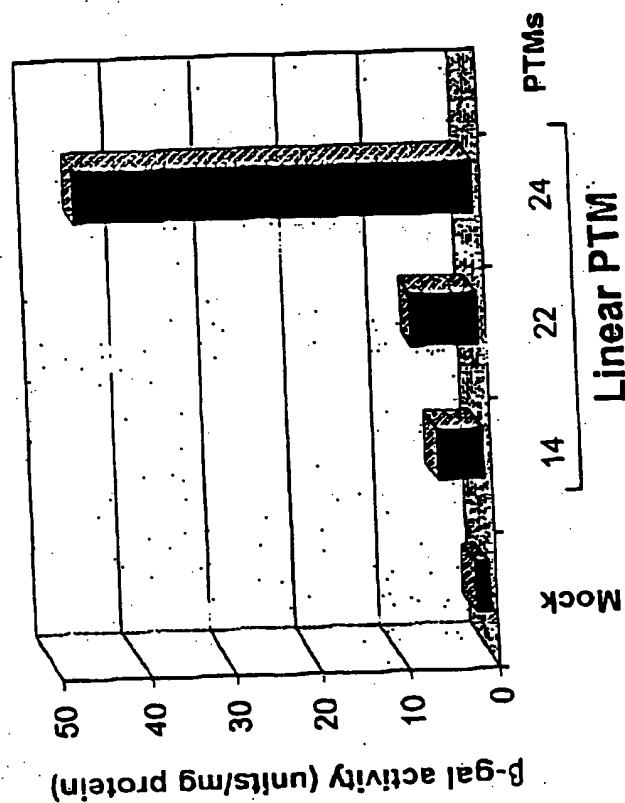


Figure 40C

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A

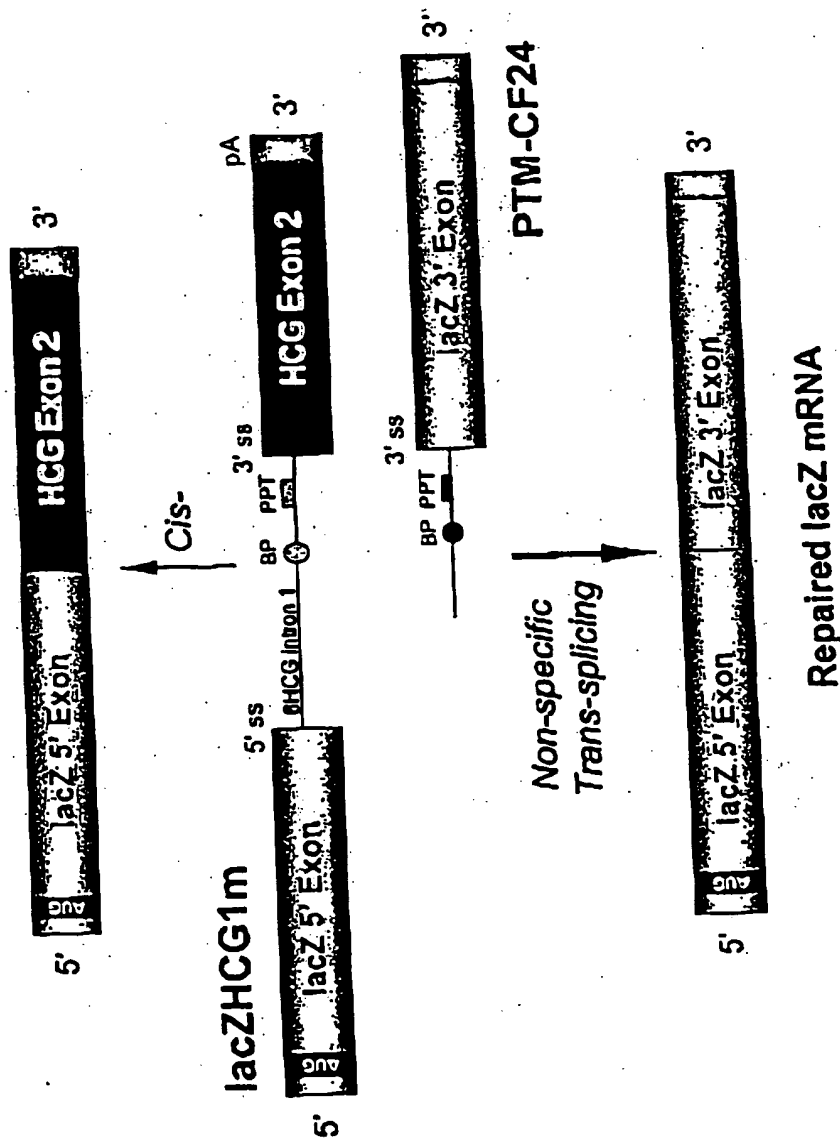


Figure 41A

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B

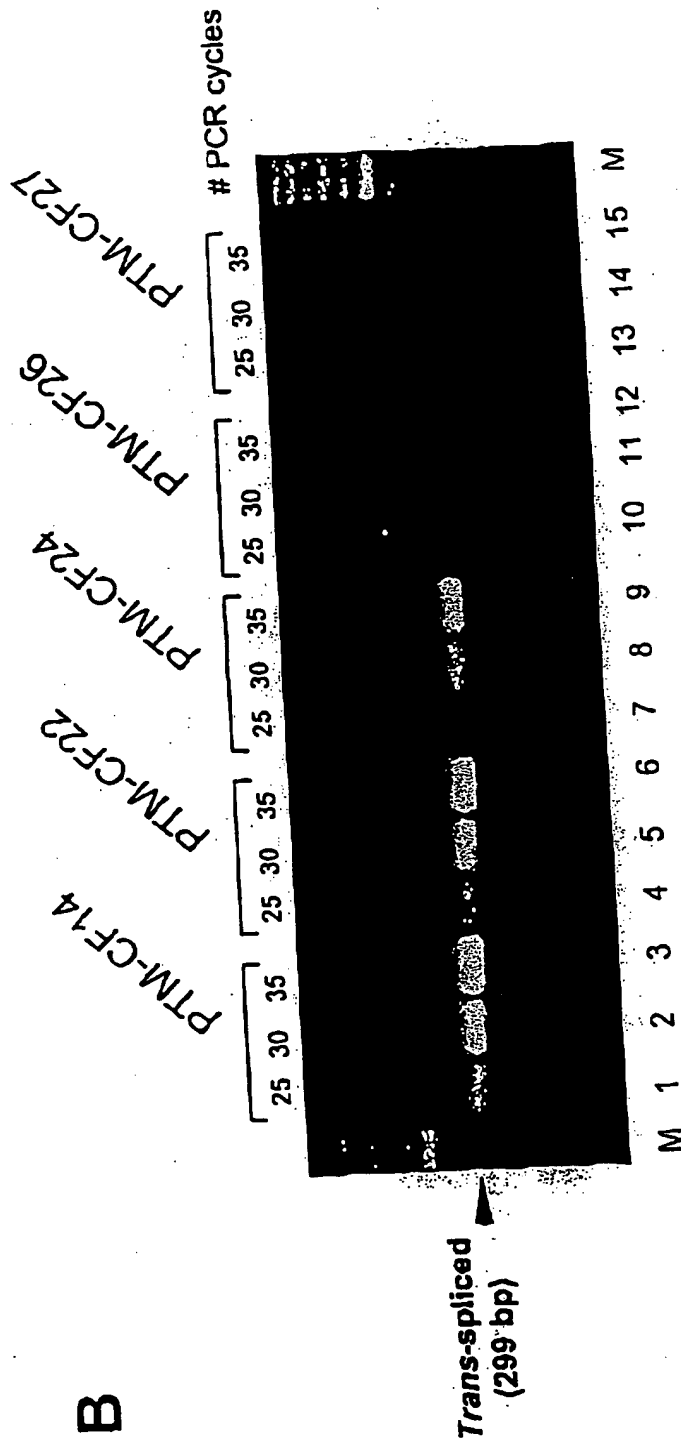


Figure 4B

C

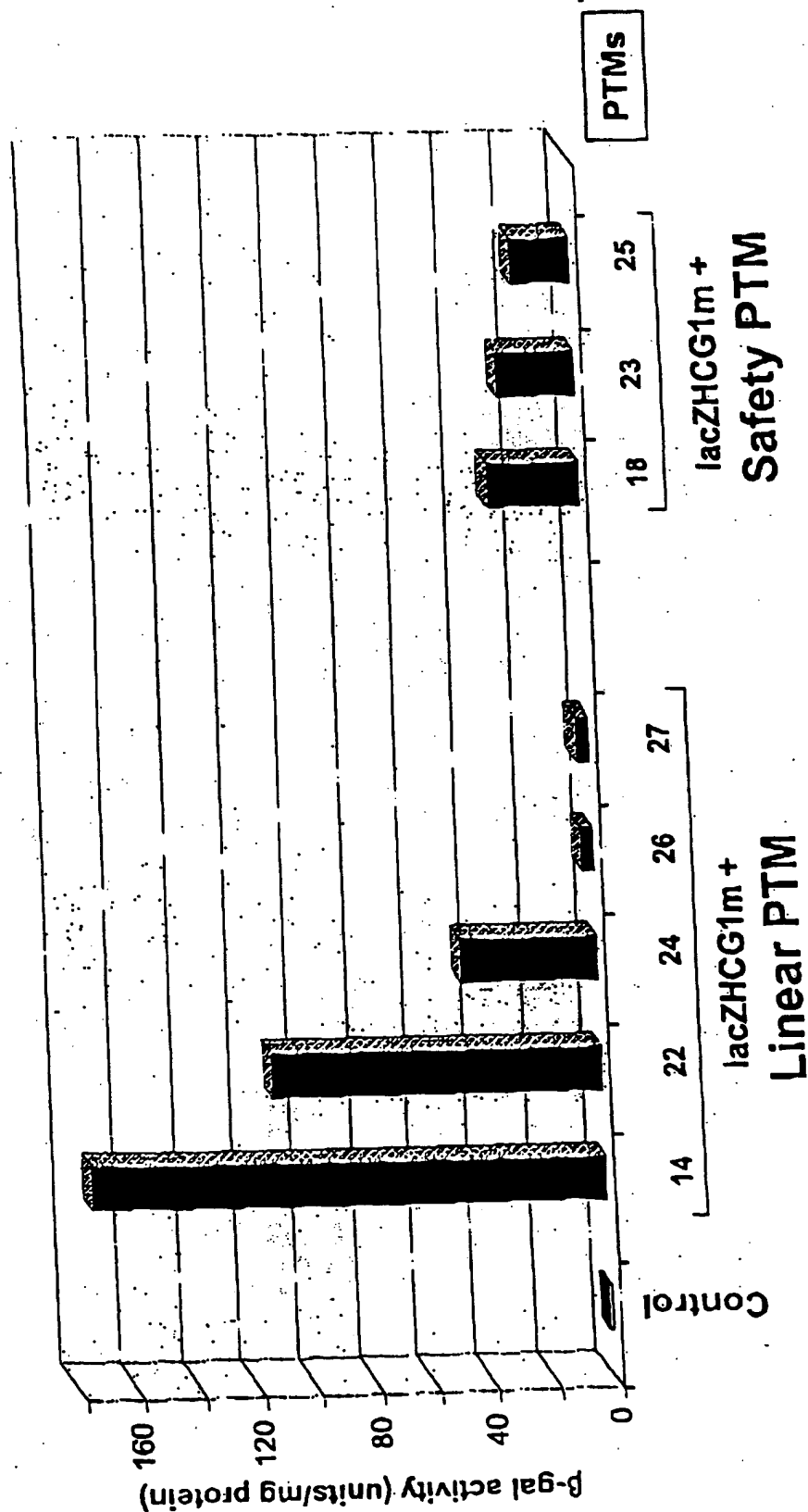


Figure 41C

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Exons 1-10

ATGCAGAGGTGCGCCTCTGGAAAAGGCCAGCGTTGTCTCCAACTTTTTTTCAGCTGGACCAGACCAATTTTGAGGAAAG
 GATACAGACAGCGCCTGGAATTGTCTAGACATATACCAATCCCTTCTGTTGATTCTGCTGACAATCTATCTGAAAAATT
 GGAAAGAGAATGGGATAGAGAGCTGGCTTCAAAGAAAAATCCTAACTCATTAAATGCCCTTCGGCGATGTTTTTCTGG
 AGATTTATGTTCTATGGAATCTTTTATATTTAGGGGAAGTCACCAAGCAGTACAGCCTCTCTTACTGGGAAGAATCA
 TAGCTTCCTATGACCCGGATAACAAGGAGGAACGCTCTATCGCGATTTATCTAGGCATAGGCTTATGCCCTTCTCTTTAT
 TGTGAGGACACTGCTCCTACACCCAGCCATTTTTTGGCCTTCATCACATTGGAATGCAGATGAGAATAGCTATGTTTAGT
 TTGATTTATAAGAAGACTTTAAAGCTGTCAAGCCGTGTTCTAGATAAAATAAGTATTGGACAACCTGTTAGTCTCCTTT
 CCAACAACCTGAACAAATTTGATGAAGGACTTGCAATTGGCACATTTTCGTGTGGATCGCTCCTTTGCAAGTGGCACTCCT
 CATGGGGCTAATCTGGGAGTTGTTACAGGCGTCTGCCTTCTGTGAGCTTGGTTTCCTGATAGTCCTTGCCCTTTTTTTCAG
 GCTGGGCTAGGGAGAATGATGATGAAGTACAGAGATCAGAGAGCTGGGAAGATCAGTGAAAGACTTGTGATTACCTCAG
 AAATGATCGAGAACATCCAATCTGTTAAGGCATACTGCTGGGAAGAAGCAATGGAAAAAATGATTGAAAACTTAAGACA
 AACAGAACTGAAACTGACTCGGAAGGCAGCCTATGTGAGATACTTCAATAGCTCAGCCTTCTTCTTCTCAGGGTTCTTT
 GTGGTGTTTTTATCTGTGCTTCCCTATGCACTAATCAAAGGAATCATCTCCGGAAAAATATTACCACCATCTCATTCT
 GCATTGTTCTGCGCATGGCGGTCACTCGGCAATTTCCCTGGGCTGTACAAACATGGTATGACTCTCTTGGAGCAATAAA
 CAAAATACAGGATTTCTTACAAAAGCAAGAATATAAGACATTGGAATATAACTTAACGACTACAGAAGTAGTGATGGAG
 AATGTAACAGCCTTCTGGGAGGAGGGATTGGGGAATTATTGAGAAAGCAAAACAAACAATAACAATAGAAAAACTT
 CTAATGGTGATGACAGCCTCTTCTTCACTAATTTCTCACTTCTTGGTACTCCTGTCTGAAAGATATTAATTTCAAGAT
 AGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACGAGCTTGGCTCATGATGATCATGGGGGAG
 TTAGAACCAGTGAAGGCAAGATCAAACTTCCGGCCGCATCAGCTTTTGAGCCCAATTCACTTGGATCATGCCCGGTA
 CCATCAAGGAGAACATAATCTTGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAAGGCCTGTCACTTGA
 GGAG

Trans-splicing domain

GTAAGATATCACCGATATGTGTCTAACCTGATTGGGCCTTCGATACGCTAAGATCCACCGG
 TCAAAAAGTTTTACATAAATTTCTTACCTCTTCTTGAATTCATGCTTTGATGACGCTTCTGTATCTATATTCATCATTG
 GAAACACCAATGATATTTCTTAAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATTTCTCCACTGT
 GCTTAATTTTACCCTCTGAATTTCCATTTCTCCATAATCATCATTACAACCTGAACCTCTGGAAATAAAACCCATCATT
 ATTAACCTCATTATCAAATCACGCT

SCANNED, #

Figure 42

153 bp PTM24 Binding Domain:

Nhe I

153 bp BD underlined

GCTAGC-XXXXXXXXXXGACGAAGCGCGCCCTCACGCTCAGGATTCACTTGCCTCCAATTATCATCCTAAGCAGAAGTGTATA

TTCTTATTTGTAAAGATTCTATTAACTCATTTGATTCAAAAATATTTAAAAATACTTCCTGTTTCACCTACTCTGCTATGC

Sac II

AC-CCGCGG

68 7 09

Figure 43A

Trans-splicing domain
AATAATGACGAAGCCGCCCTCACGCTCAGGATTCACTTGCCCTCCAATTATCATCCTAAGCAGAAGTGTATATTCTTA
TTTGTAAGATTCTATTAACTCATTTGATTCAAAATATTTAAATACTTCTGTTCACCTACTCTGCTATGCACCCGC
GGAACATTATTATAACGTTGCTCGAATACTAACTGGTACCTCTTCTTTTTTTTGATATCTGCAG

Exons 10-14

ACTTTCACCTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAATTAAGCACAGTGGAAGAATTTCACTTCT
GTTCTCAGTTTTTCTGGATTATGCTTGGCACCATTAAAGAAAATATCATCTTTGGTGTTCCTATGATGAATATAGATA
CAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGGACATCTCCAAGTTTGAGAGAAAGACAATATAGTTCCTGGAGAA
GGTGAATCACACTGAGTGGAGGTCAACGAGCAAGAATTTCTTTAGCAAGAGCAGTATACAAAGATGCTGATTGTATT
TATTAGACTCTCCTTTTGATACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTGTCTGTAACATGATGGC
TAACAAAACCTAGGATTTTGGTCACTTCTAAAATGGAACATTTAAAGAAAGCTGCACAAAATATTAATTTTGCATGAAGGT
AGCAGCTATTTTTATGGGACATTTTTCAGAACTCCAAATCTACAGCCAGACTTTAGCTCAAACCTCTGGGATGTGATT
CTTTTCGACCAATTTAGTGAGAGAAAGAAATTCCTAATCTAAGTACAGCTTACACCGTTTTCTCATTAGAAGGAGATGC
TCCTGTCTCCTGGACAGAAACAAAAACAATCTTTTAAACAGACTGGAGAGTTTGGGAAAAAGGAAGAATTCCTATT
CTCAATCCAATCAACTCTATACGAAAATTTTCATTGTGCAAAAGACTCCCTTACAAATGAATGGCATCGAAGAGGATT
CTGATGAGCCTTTAGAGAGAAGGCTGTCTTAGTACCAGATTCTGAGCAGGGAGAGGCGATACCTCCCTCGCATCAGCGT
GATCAGCACTGGCCCCACGCTTCAGGCAGGAAGGAGGAGCTGTCTGTGAGACCTGATGACACACTCAGTTAAACCAAGGT
CAGAACATTCACCGAAGACAACAGCATCCACAGAAAAGTGTCACTGGCCCCCTCAGGCACAACTTGACTGAACTGGATA
TATATTCAAGAAGGTTATCTCAAGAACTGGCTTGGAAATAAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTT
TTTTGATGATATGGAGAGCATACCAGCAGTGACTACATGGAACACATACCTTCGATATATTACTGTCCACAAGAGCTTA
ATTTTTGTGCTAATTTGGTGTCTAGTAATTTTTCTGGCAGAGGTGGCTGCTTCTTTGGTGTGTGTGGCTCTCTGGAA
ACACTCCTCTTCAAGACAAAGGGAATAGTACTCATAGTAGAAATAACAGCTATGTCAGTGATTATCACCGACCCAGTTG
GTATTATGTGTTTTACATTTACGTGGGAGTAGCCGCACTTTGCTTGTATGGAATTTCTAGAGTTCTCAGAGGTTCTACCACTGGTG
CATACTCTAATACAGTGTGCAAAATTTTACACCACAAAATGTTACATTTCTGTTCTTCAAGCACCTATGTCAACCCTCA
ACAGTGTGAACAGGTCAGGTGGGATTCTTAATAGATTCTCCAAAGATATAGCAATTTTGGATGACCTTCTGCCTCTTACCAT
ATTTGACTTCATCCAGTTGTTATTAATTGTGATTGGAGCTATAGCAGTTGTGCGAGTTTTTCAACCCTACATCTTTGTT
GCAACAGTGCCAGTGATAGTGGCTTTTATTATGTTGAGAGCATATTTCTCCAAACCTCACAGCACTCAACCAACTGG
AATCTGAAGGCAGGAGTCCAATTTTCACTCATCTTGTTACAAGCTTAAAGGACATGAGACATTCGTGCCTTCGGACG
GCAGCCTTACTTTGAAACTGTGTTCCACAAAGCTCTGAATTTACATATGCTGCGCACTGGTTCTTGTACCTGTCAACACTG
CGCTGGTTCCAAATGAGAATCTGAAATGATTTTTGTCTACTTTCTTCATTGCTGTTACCTTCATTTCCATTTTAAACAAG
GAGAAGGAGAAGGAAGAGTTGGTATTATCTGACTTTAGCCATGAATATCATGAGTACATTGCAGTGGGCTGTAAACTC
CAGCATAGATGTGGATAGCTTGATGCGATCTGTGAGCCGAGTCTTTAAGTTCATTGACATGCCAACAGAAGGTAAACCT
ACCAAGTCAACCAAAACCATACAAGAATGGCCAACTCTCGAAAGTTATGATTATTGAGAATTCACAGCTGAAGAAGATG
ACATCTGGCCCTCAGGGGGCCAAATGACTGTCAAAGATCTCACAGCAAAATACAGAAAGGTGGAATGCCATATTAGA
GAAACATTTCTTCTCAATAAGTCTCTGGCAGAGGGTGGGCCTCTGGGAAGAACTGGATCAGGGAAGAGTACTTTGTTA
TCAGCTTTTTTGGAGACTACTGAAACACTGAAGGAGAAATCCAGATCGATGGTGTGTCTTGGGATTCAATAACTTTGCAAC
AGTGGAGGAAAGCCTTTGGAGTGATACCACAGAAAGTATTTATTTTTCTGGAACTTTAGAAAAAACTTGGATCCCTA
TGAACAGTGGAGTGATCAAGAAATATGGAAGTTGCAGATGAGGTTGGGCTCAGATCTGTGATAGAACAGCTTTCTGGG
AAGCTTGACTTTGTCTTTGTGGATGGGGGCTGTGTCTTAAGCCATGGCCACAGCAGCTGTGATGTGCTGGCTAGACTGT
TTCTCAGTAAGGCAGAGATCTGTCTGTGATGAACCCAGTGTCTATTTGGATCAGTAACATACCAATAATTAGAAG
AACTCTAAAAACAGCAATTTCTGTGATTGCGCAGTAATTTCTGTGAAACACAGGATAGAAGCAATGTCTGGAATGCCAACAA
TTTTTGGTCATAGAAGAGAACAAAGTGCGGCAGTACGATTCATCCAGAAACTGCTGAACGAGAGGAGCCTCTTCCGGC
AAGCCATCAGCCCTCCGACAGGGTGAAGCTCTTCCCCACCGGAACCTCAAGCAAGTGCAAGTCAAGCCCGAGATTGC

Histidine tag Stop

TGCTCTGAAAGAGGAGACAGAAGAAGAGGTGCAAGATACAAGGCTTCATCATCATCATCATTAG

Figure 43B

68 J 79



Figure 44 A

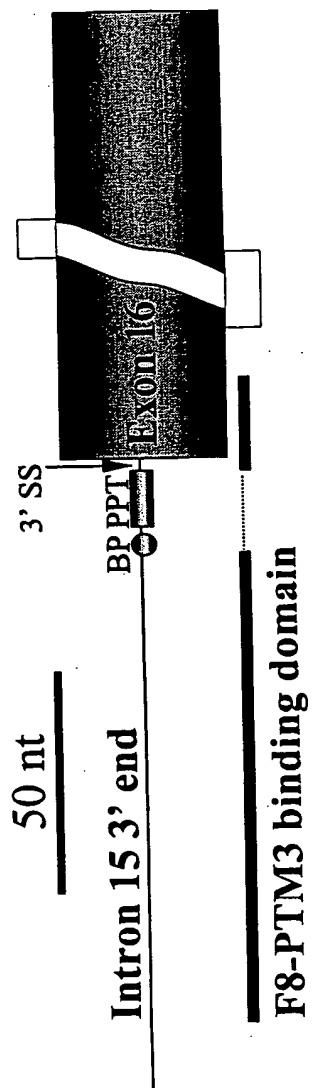
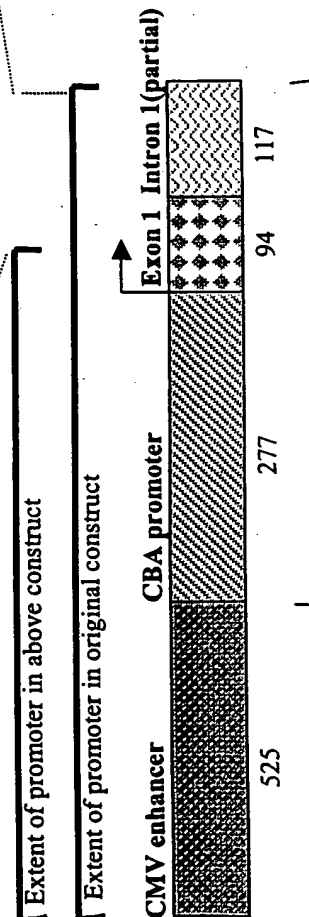


Figure 44B

Figure 44C

NNnucleotide changes are shown in blue
Boxed = CAT box, TATA box
Boxed + Arrow = Transcription Start
Oval = Downstream elements
Bold = Binding domain
Italicized = Spacer+PPT+BP+AG dinucleotide

Sequence not included in construct

$$\begin{aligned} \mathbf{F13} + \mathbf{F2} &= 235 + 106 = 341 \text{ bp} \\ \mathbf{F13} + \mathbf{F4} &= 235 + 315 = 550 \text{ bp} \end{aligned}$$


Chicken Beta Actin Promoter (including exon 1 and part of intron 1)

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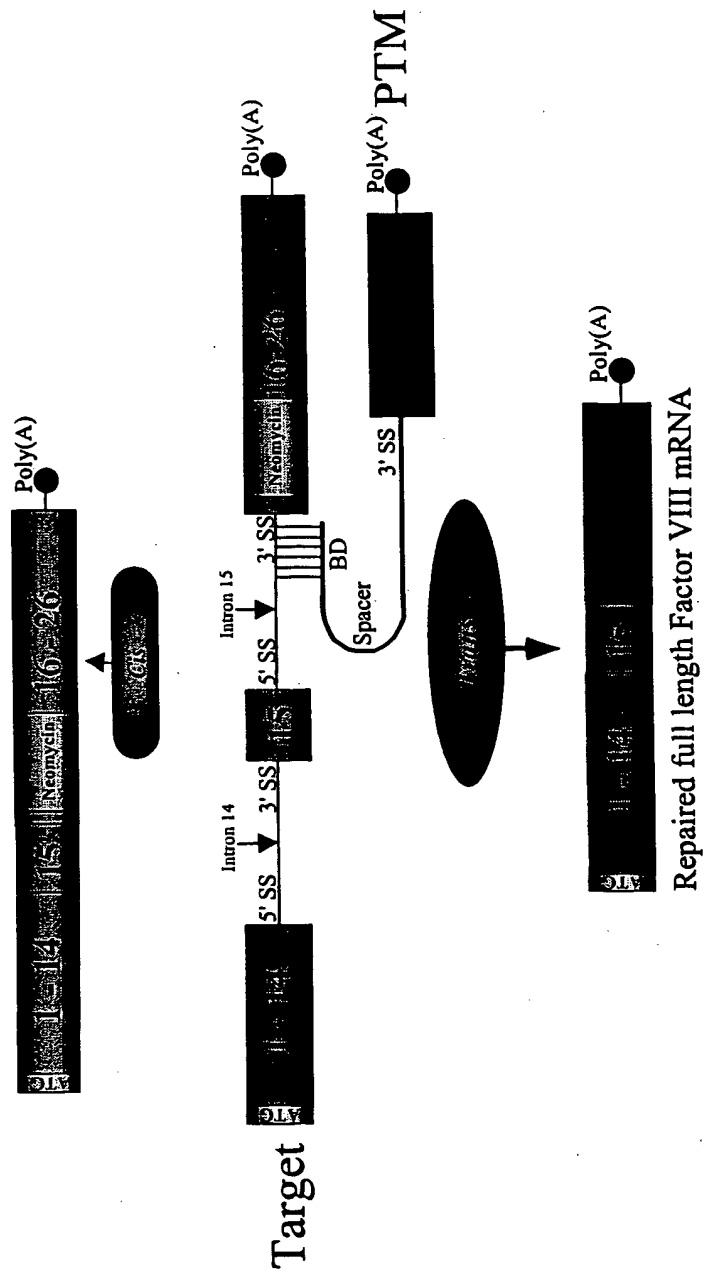
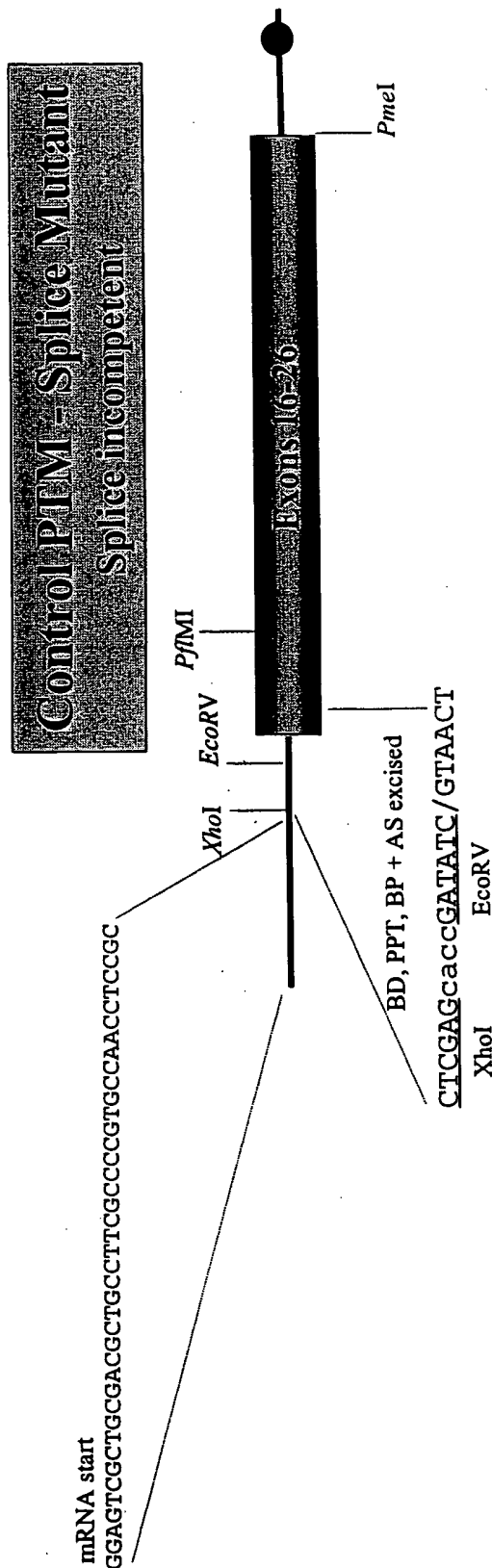


Figure 44D

Figure 45



Method:

Excise TSD and part of exon 16 with XhoI and PfuMI and ligate in a PCR product that:

- 1) eliminates the TSD and splice acceptor site
- 2) inserts EcoRV adjacent to exon 16
- 3) restores the coding for exon 16

Repair of Factor VIII

Preliminary results from one experiment

FVIII activity in Exon 16 FVIII-KO mice
after IV PTM-FVIII intraportal infusion
(100ugDNA)(n=3)

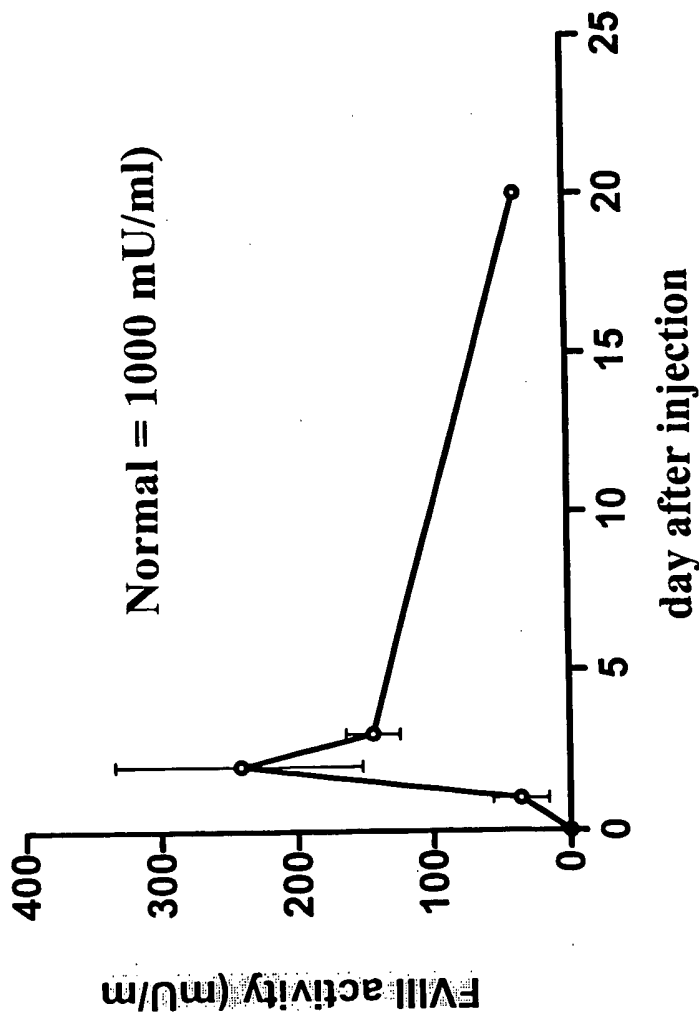


Figure 46

METHODS

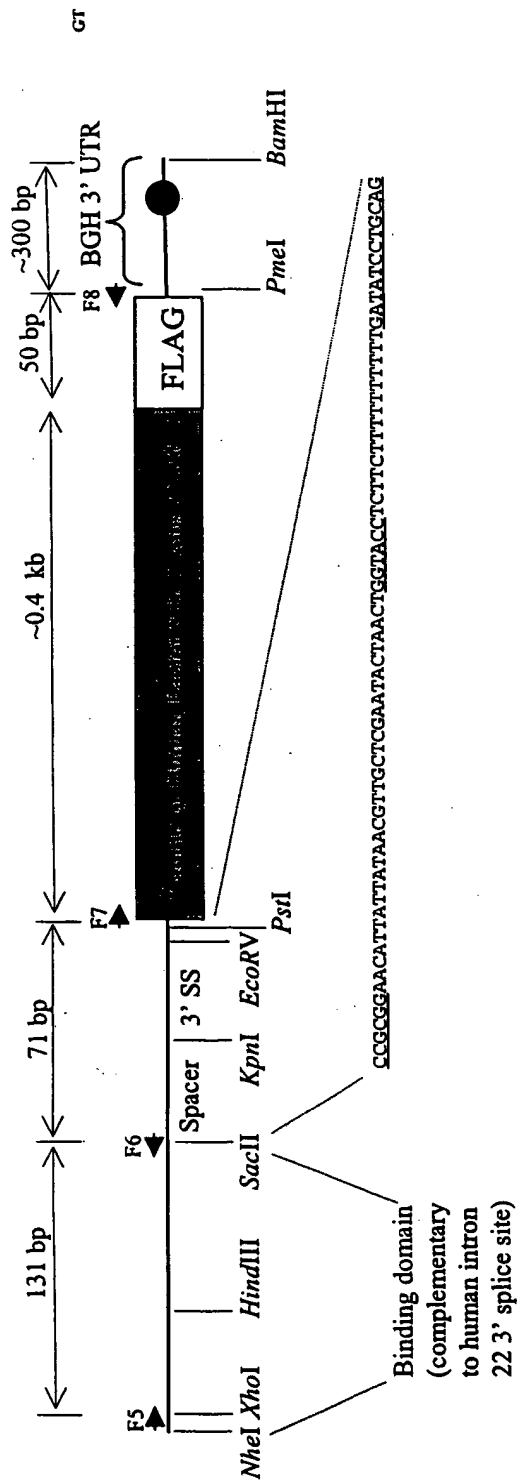
Inject plasmid intraportally



Sample blood (1, 2, 3, 20 d)



Assay for factor VIII activity



FLAG = C-terminal tag to be used to detect repaired factor VIII protein.

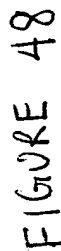
Figure 47B

THE UNIVERSITY OF CHICAGO



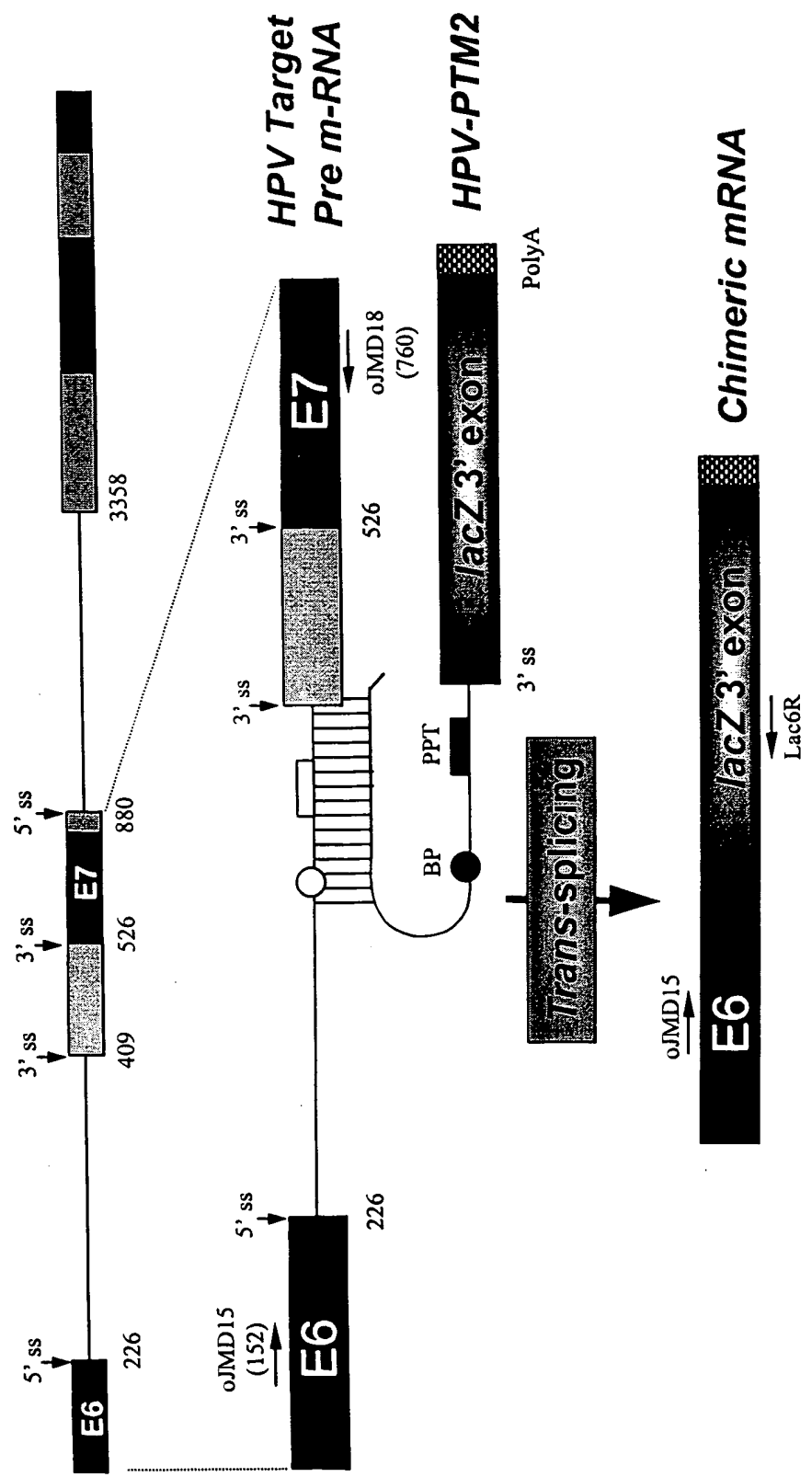
2

02



SMART Strategy to Disrupt the Expression of Human Papillomavirus Type 16

Targeting E6 Exon



SMART Strategy by 3' Exon Replacement: Schematic diagram of HPV-PTM2 binding to the 3' splice site of the HPV type 16 target pre-mRNA

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PTM Design

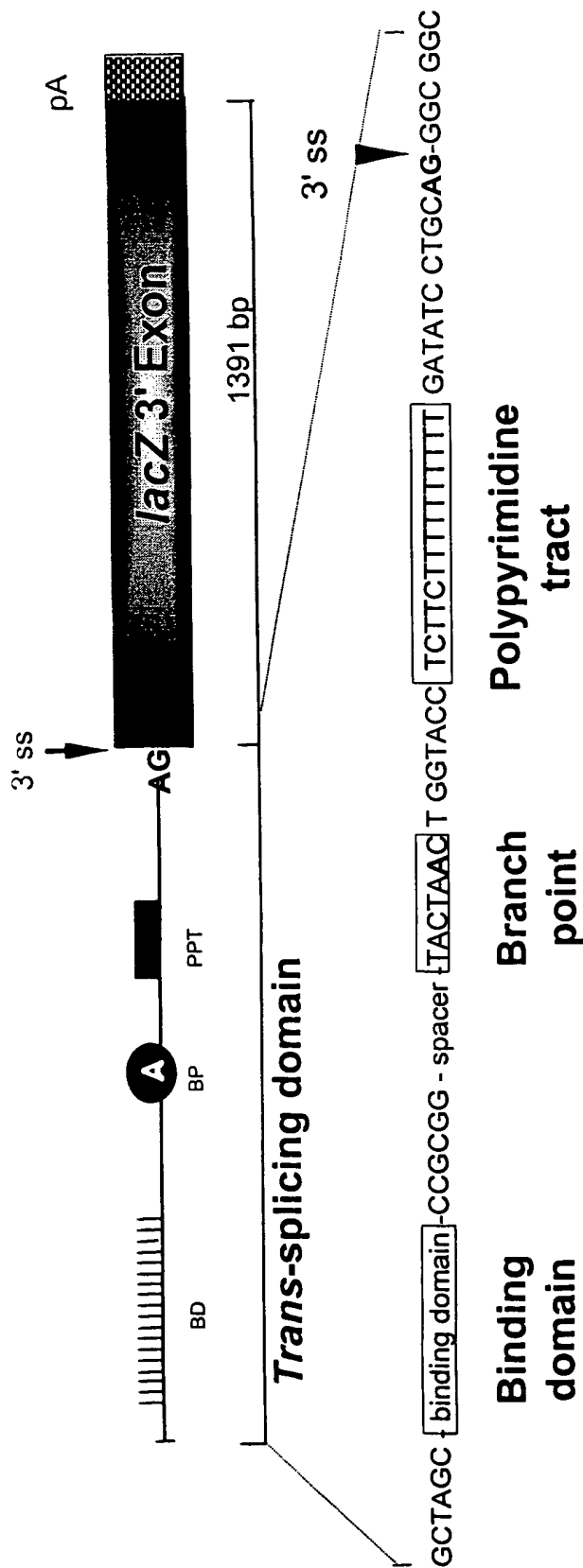
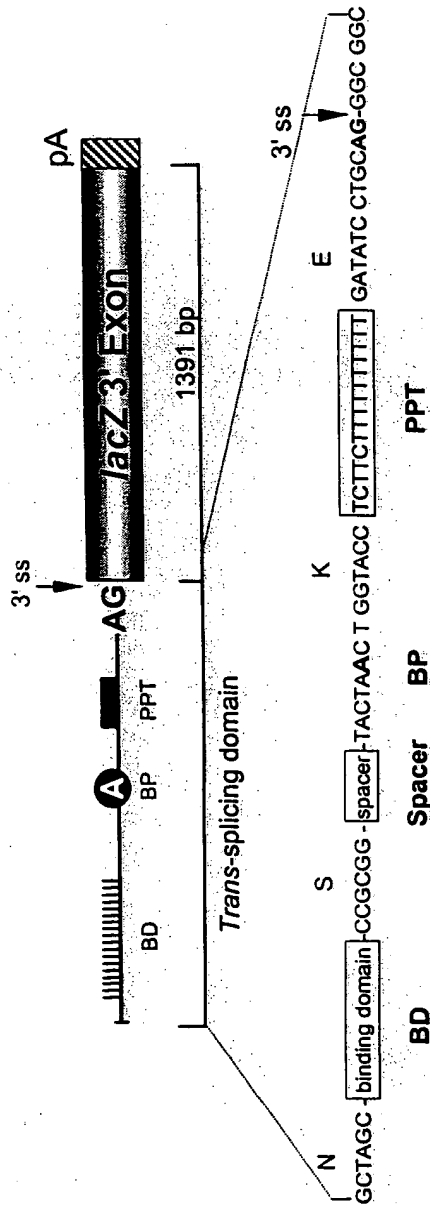


FIGURE 5/

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HPV-PTM1 with 80 bp binding domain targeted to 3' ss at 409 :



Binding domain sequence: CAGTTAATAC ACCTAATTAA CAAATCACAC AACGCTTTGT TGTATTGCTG
TTCTAATGTT GTTCCATACA CACTATAACA

HPV-PTM2 with 149 bp binding domain targeted to 3' ss at 409:

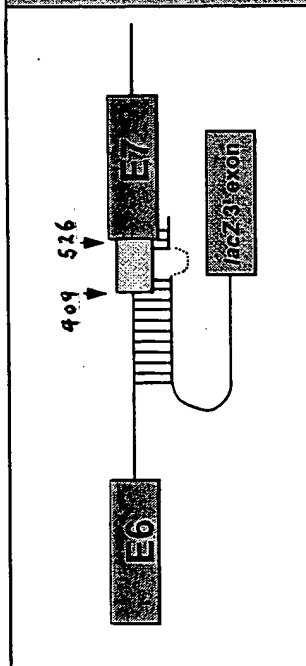


Binding domain sequence: CAGTTAATAC ACCTAATTAA CAAATCACAC AACGCTTTGT TGTATTGCTG
TTCTAATGTT GTTCCATACA CACTATAACA ATAATGTCTA TACTCACTAA
TTTTAGAATA AAACCTTTAA CATTATCAC ATACAGCATA TCGATTCCC

Binding Domains of HPV-PTM3 and 4

HPV-PTM3 Binding domain (covers both 3' ss at 409 and 526; has 53 bp bubble)

GATGATCTGCAACAAGACATACATCGACCGGTCCA (53 nt bubble) CTTACGACACAGTGGCTTTTGAC
AGTTAAATACACCTAATTAAACAAATCACACAAACGGTTTGTGTATTCAGTTCTTAATGTTGCCATACACACTA
TAACAAAT



HPV-PTM4 Binding domain (covers both 3' ss at 409 and 526; has 76 bp bubble)

GATGATCTGCAACAAGAC (76 nt bubble) GACACAGTGGCTTTTGACAGTTAAATACACCTAAATTAAACAAATC
ACACAAACGGTTTGTGTATTCAGTTCTTAATGTTGCCATACACACTATAACAAT

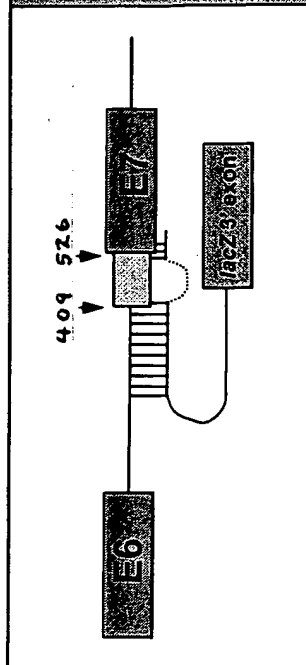


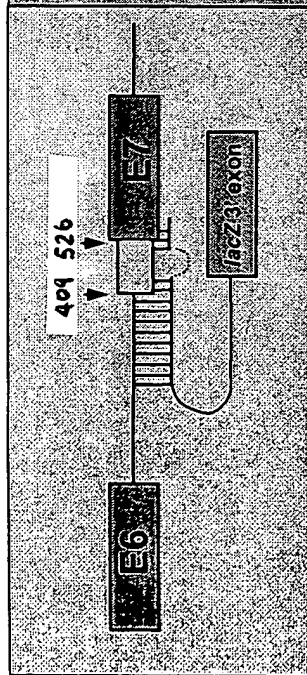
FIGURE 5.3

HPV-PTM5 and 6

HPV-PTM5, Binding domain (140 nt, has 53 nt bubble, covers 3'ss at position 409 and 526)

GATGATCTGCAACAAGACATACATCGAC**CCGGT**CCA. CTTGAGGACACAGTGGCTTTTGACAGTTAATACACCTAATTAAACAATACACAA**CCGGT**
TTGTTGTATTGCAGTTCTAATGTTGTTCCATACACACTATAACA

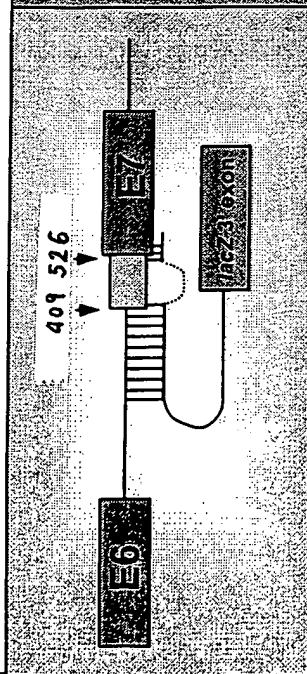
CCGT



HPV-PTM6, Binding domain (117 nt, has 76 nt bubble, covers 3'ss at position 409 and 526)

GATGATCTGCAACAAGAC.GACACAGTGGCTTTTGACAGTTAATACACCTAATTAAACAATACACAA**CCGGT**TTGTTGTATTGCAGTTCT
AATGTTGTTCCATACACACTATAACA

CCGT

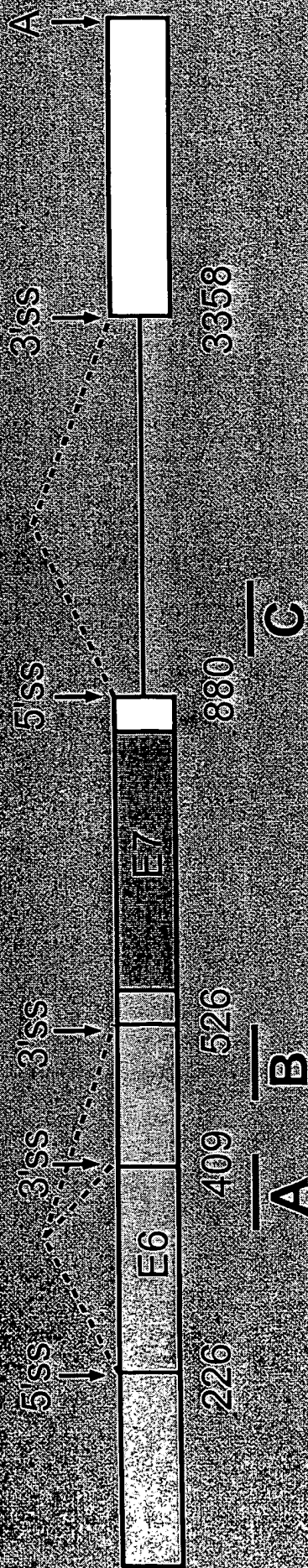


Note: Nucleotides in bold are modified to prevent PTMs cryptic splicing

FIGURE 54

INTRONN

Positions of HPV-PTM Targeting Domains

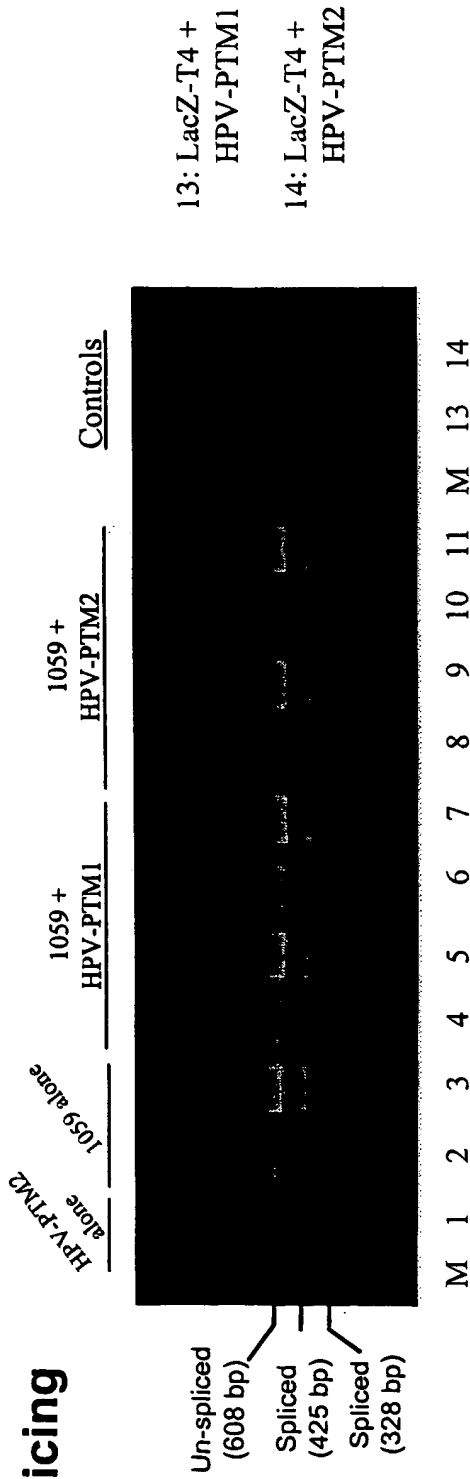


Binding Domain		
PTM	Region	Size (nt)
HPV-PTM1	A	80
HPV-PTM2	A	149
HPV-PTM5	A+B	140
HPV-PTM6	A+B	117
HPV-PTM8	C	104
HPV-PTM9	C	174

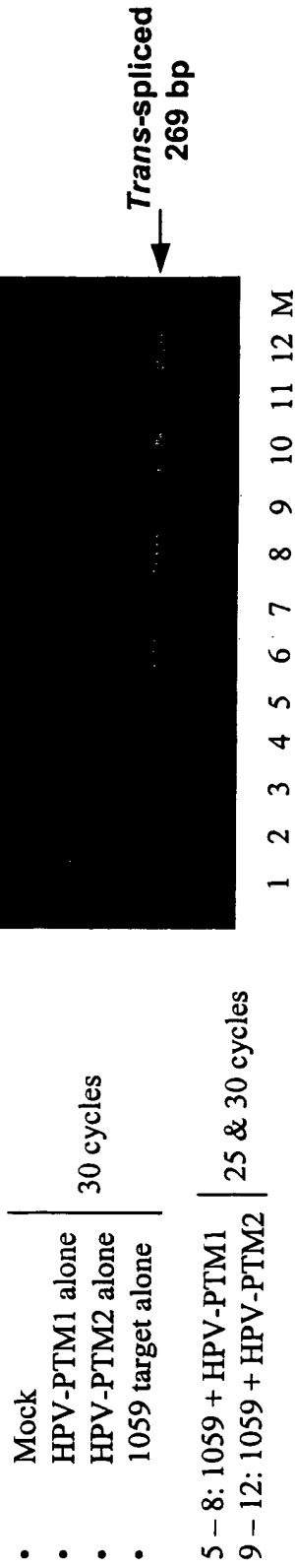
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Trans-splicing Efficiency of HPV-PTMs in 293T Cells

Cis-splicing

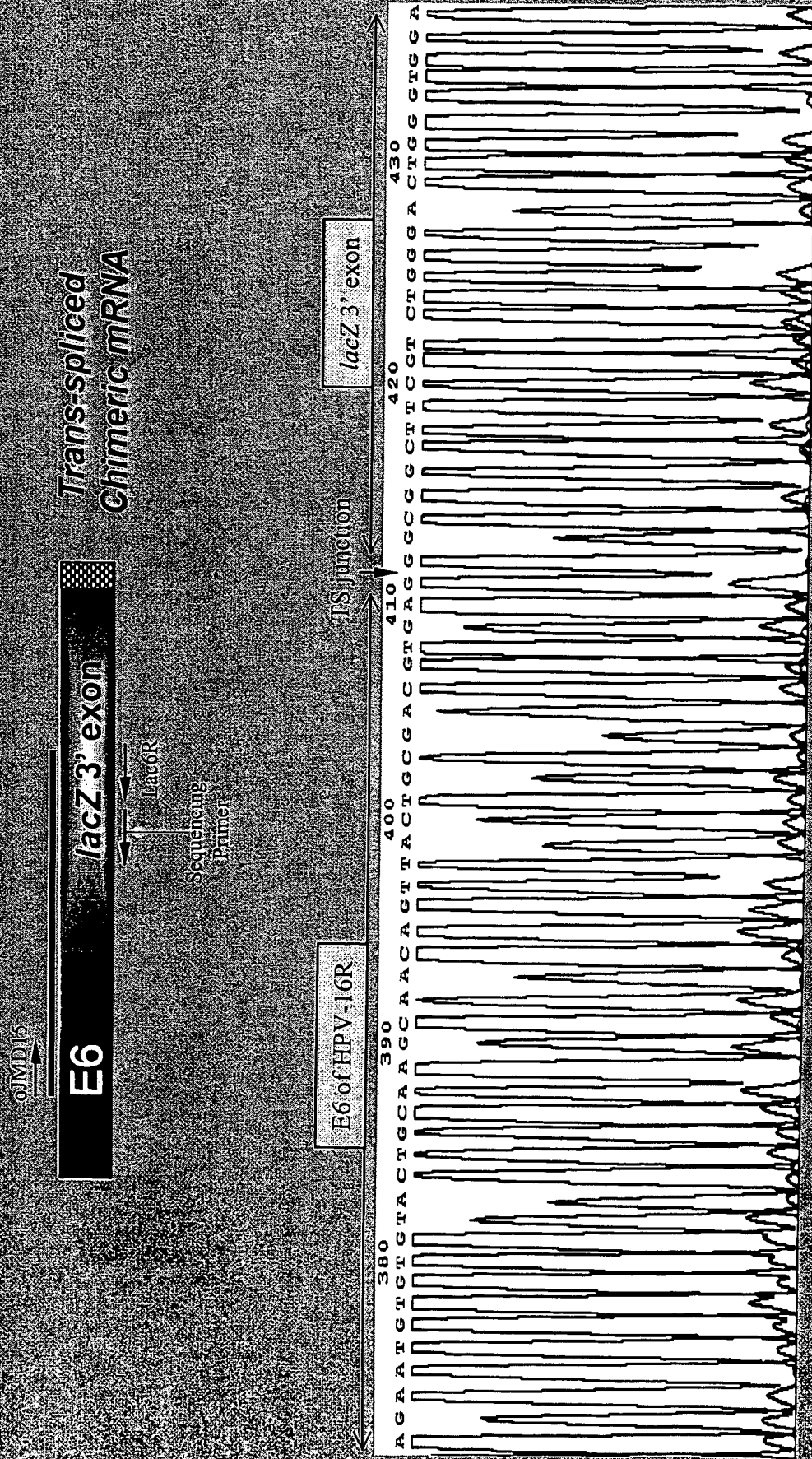


Trans-splicing



RT-PCR Analysis of total RNA

**Trans-splicing between target pre-mRNA and
PTM is accurate (293T cells)**

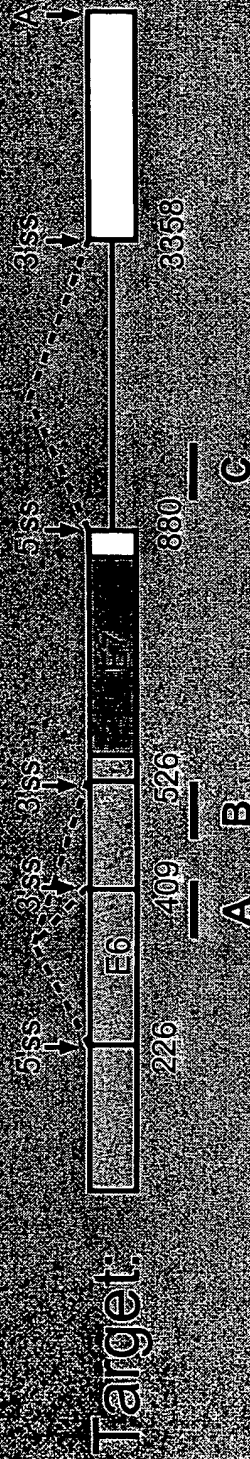


INTRON

FIGURE 57

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Trans-splicing in 293 Cells (Co-transfections)



PTM	<u>Binding Domain</u>		<u>% trans-spliced</u>	
	<u>Region</u>	<u>Size (nt)</u>	<u>226 sd</u>	<u>880 sd</u>
HPV-PTM1	A	80	69	0.6
HPV-PTM2	A	149	45	0.9
HPV-PTM5	A+B	140	55	0.8
HPV-PTM5ΔBP/PPT	A+B	140	0.5	0.2
HPV-PTM6	A+B	117	59	1
HPV-PTM8	C	104	7	37
HPV-PTM9	C	174	14	22
CF-PTM27	CF intron	411	0	0

Quantification of *trans*-splicing efficiency using real-time QRT-PCR

FIGURE 58

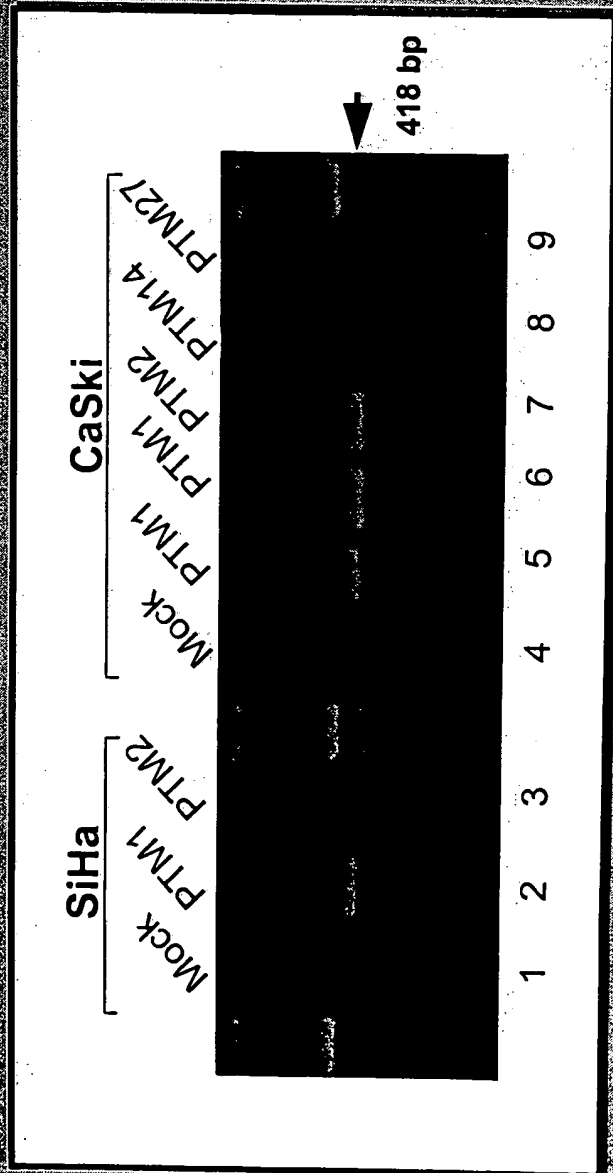
Trans-splicing into Endogenous HPV Pre-mRNA Target in SiHa & CaSki Cells

RT-PCR Conditions

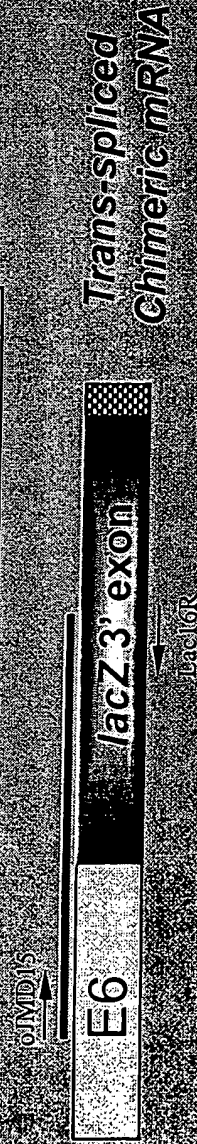
- Total RNA: 400 ng/rxn
- Primer's: oJMD15 + Lac16R
- # Cycles : 35
- Expected product : 418 bp

Details

- PTM1, PTM2 : HPV targeted, specific
- PTM14 : CF targeted, non-specific, has 23 bp BD
- PTM14 : CF targeted, non-specific, has 411 bp BD



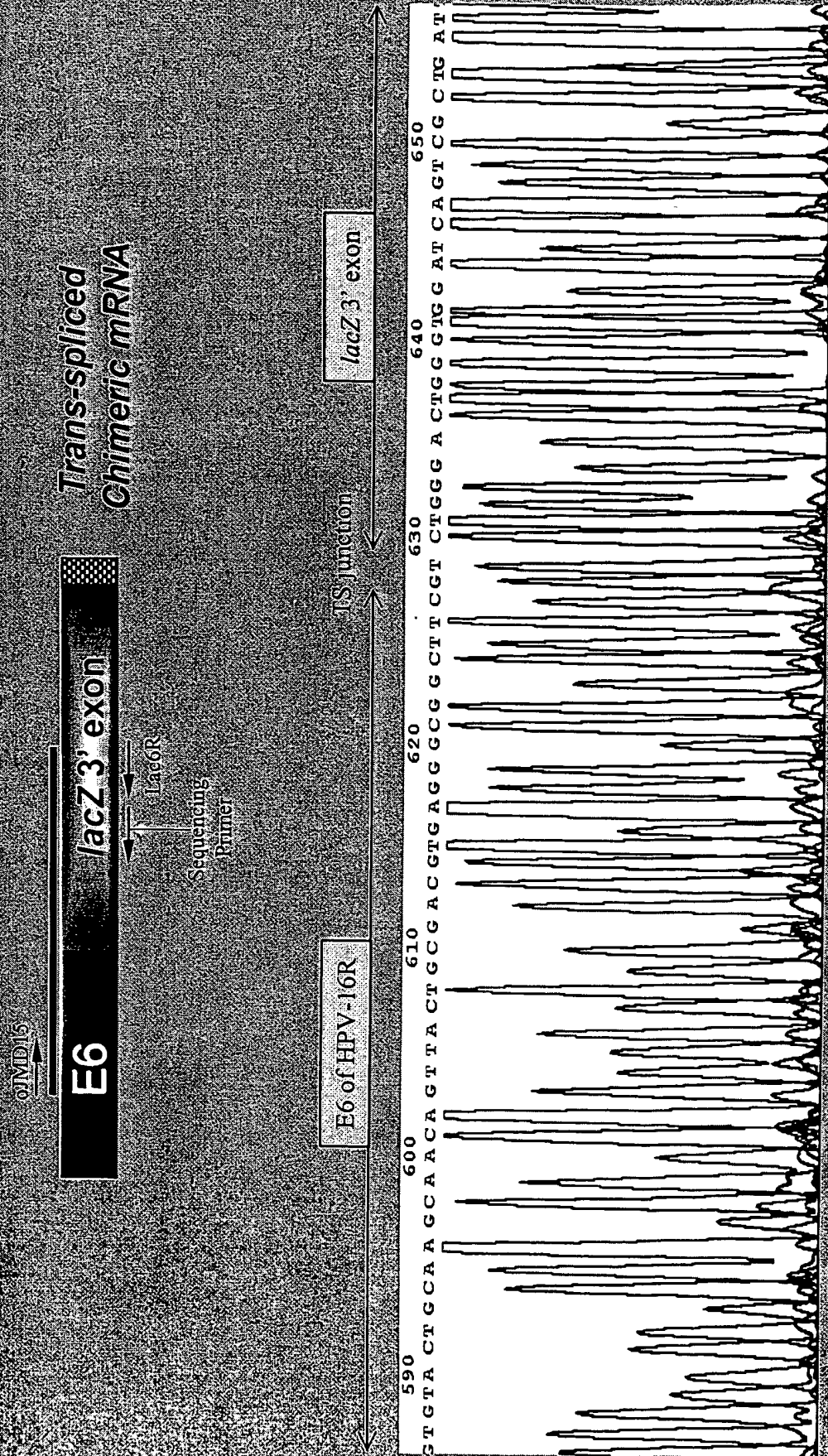
SiHa : Single copy
CaSki : ~ 400-500 copy!!



RT-PCR Analysis of total RNA

FIGURE 59

Accurate Trans-splicing of HPV-PTM1 in Si Ha Cells (Endogenous target pre-mRNA)



INTRONN

FIGURE 60

Trans-splicing in SiHa Transfections

(Endogenous target)

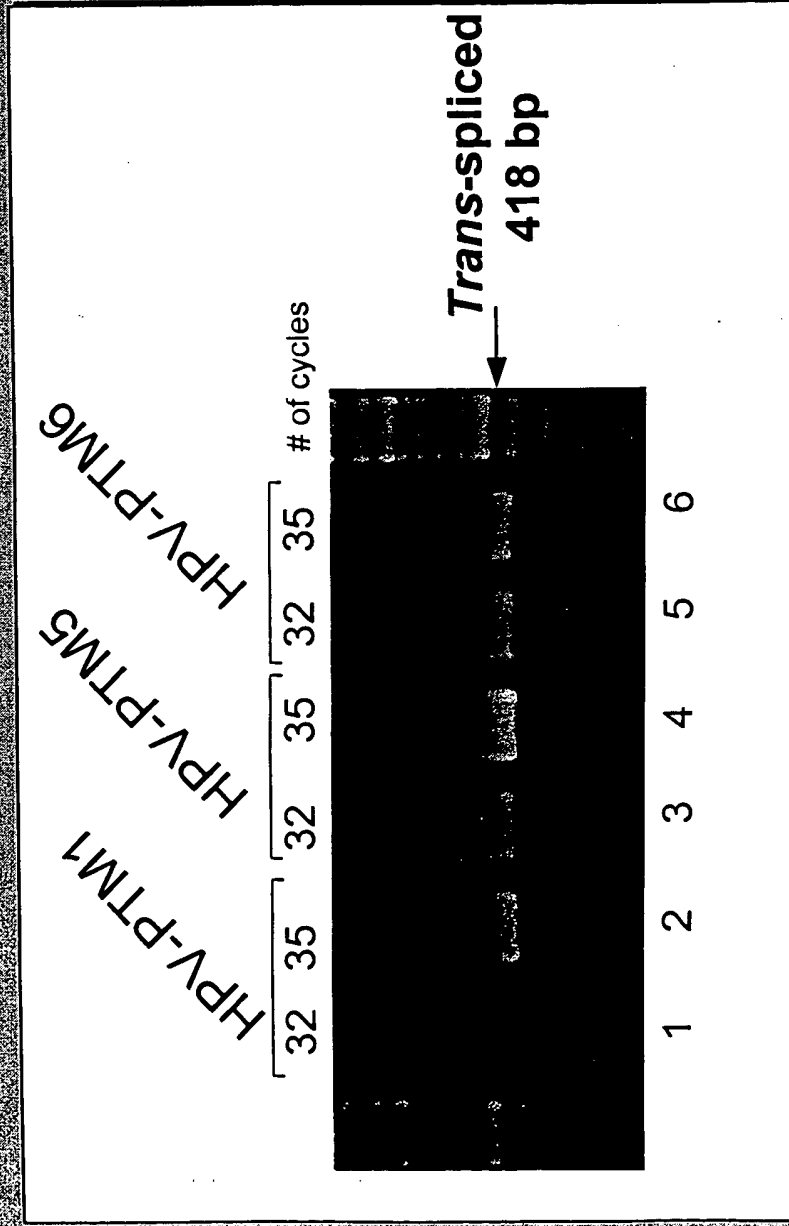
<u>PTM</u>	<u>% <i>trans</i>-spliced</u>
pcDNA3.1	0
HPV-PTM1	0.16
HPV-PTM5	0.12
HPV-PTM6	0.11
CF-PTM27	0

Quantification of *trans*-splicing efficiency using real-time QRT-PCR

INTRONN

FIGURE 61

Trans-splicing Efficiency of HPV-PTM1, 5, & 6 in SiHa Cells



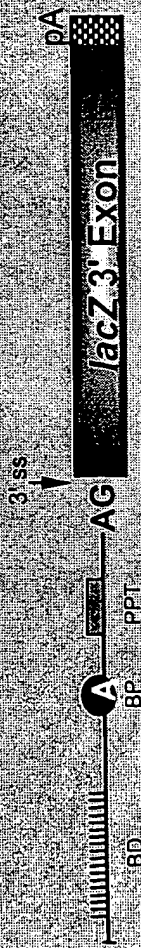
- SiHa cells transfected with 1.5 µg plasmid DNA, LipoPlus
- RNA isolated after 48 hr
- Total RNA: 500 ng/Rxn
- Primers: oJMD15 + Lac16R
- Expected product: 418 bp

1, 3, 5, : 32 cycles

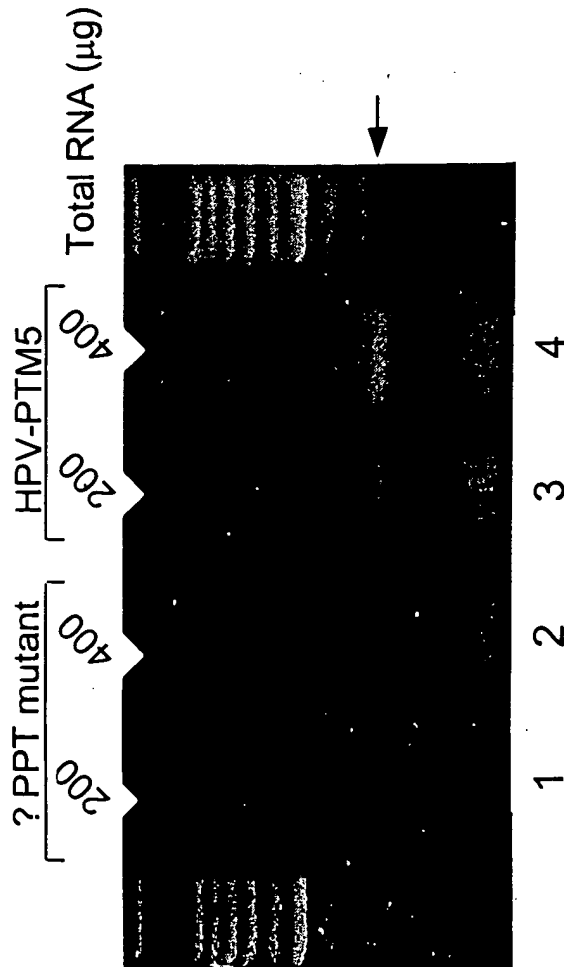
2, 4, 6, : 35

Deletion of polypyrimidine tract abolishes *trans*-splicing

HPV-PTM5 (has all the elements)



HPV-?PPT (PPT has been deleted)



Methods:

- SiHa cells transfected with 1.5 µg of plasmid DNA
- Total RNA isolated after 48 hr and analyzed by RT-PCR (30 cycles)

Primers: oJMD15+Lac6R
Expected product: 269 bp

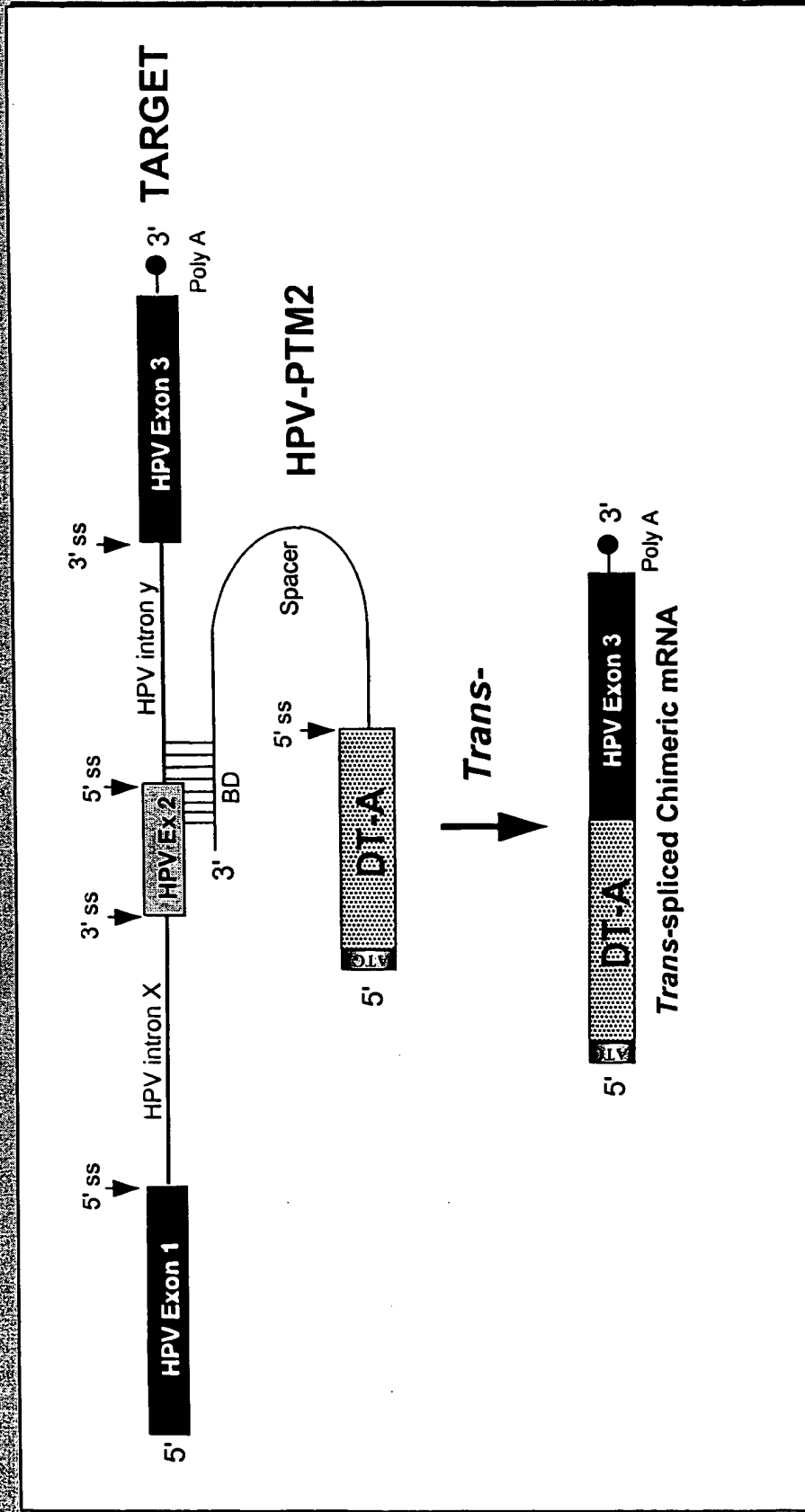
Lanes 1 & 2: RNA from cells transfected with HPV-? PPT (mutant); No trans-splicing detected

Lanes 3 & 4: RNA from cells transfected with HPV-PTM5 plasmid; trans-splicing Detected (269 bp product)

INTRONN

FIGURE 63

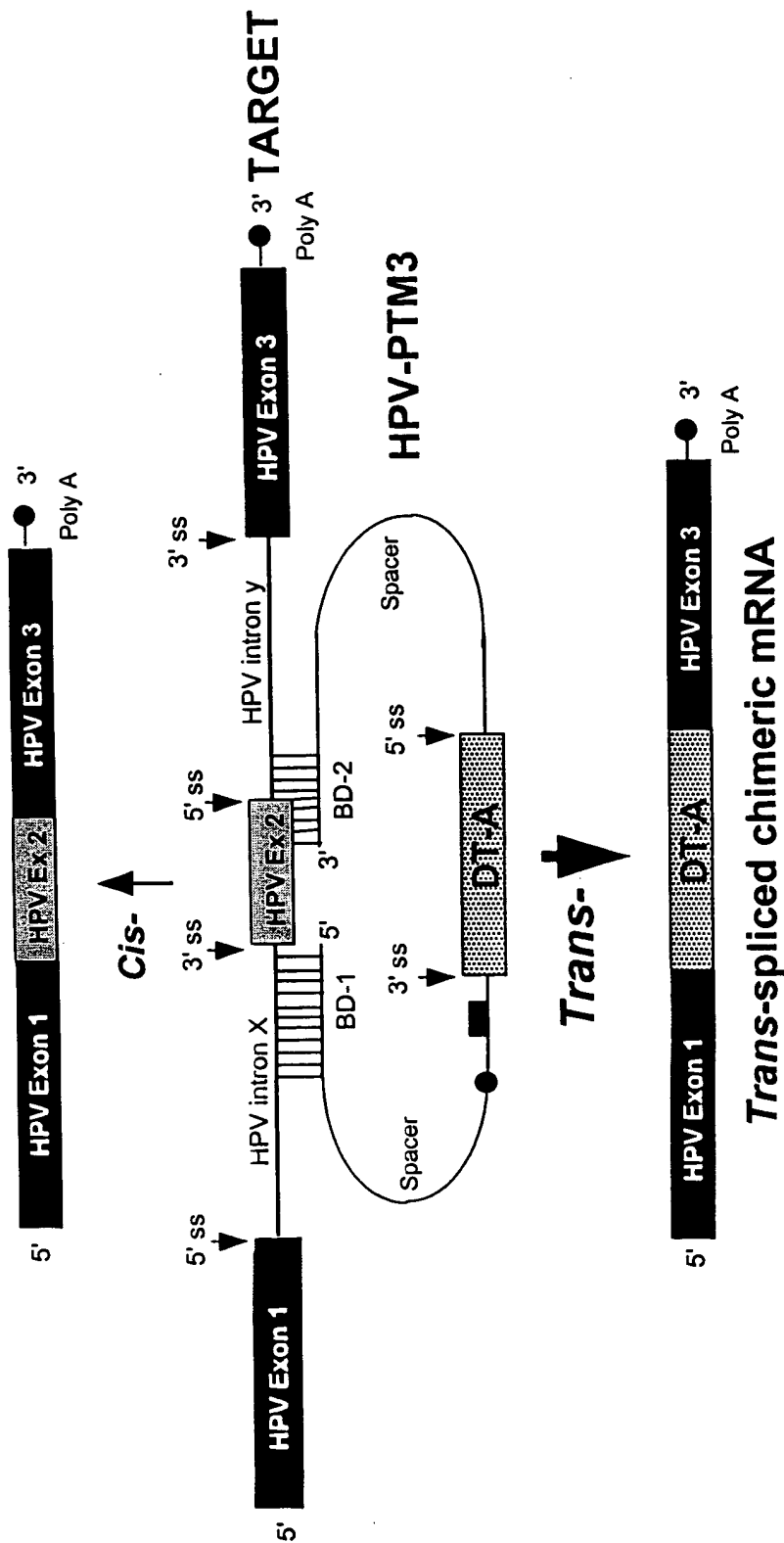
SMaRT Strategy by 5' Exon Replacement



Schematic diagram of a PTM binding to the 5' splice site of the HPV mini-gene target

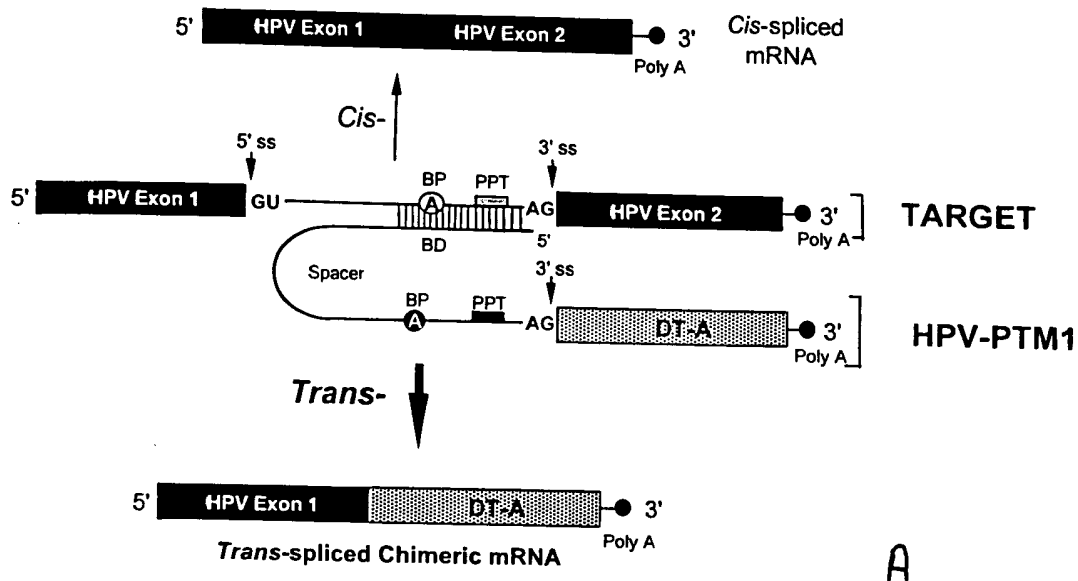
hr to 9.2

Double Trans-splicing



Schematic diagram of a double trans-splicing PTM binding to the 3' and 5' splice sites of the HPV mini-gene target

SMaRT Strategy by 3' Exon Replacement: Schematic diagram of a PTM binding to the 3' splice site of the HPV mini-gene target



SMaRT Strategy by 5' Exon Replacement: Schematic diagram of a PTM binding to the 5' splice site of the HPV mini-gene target

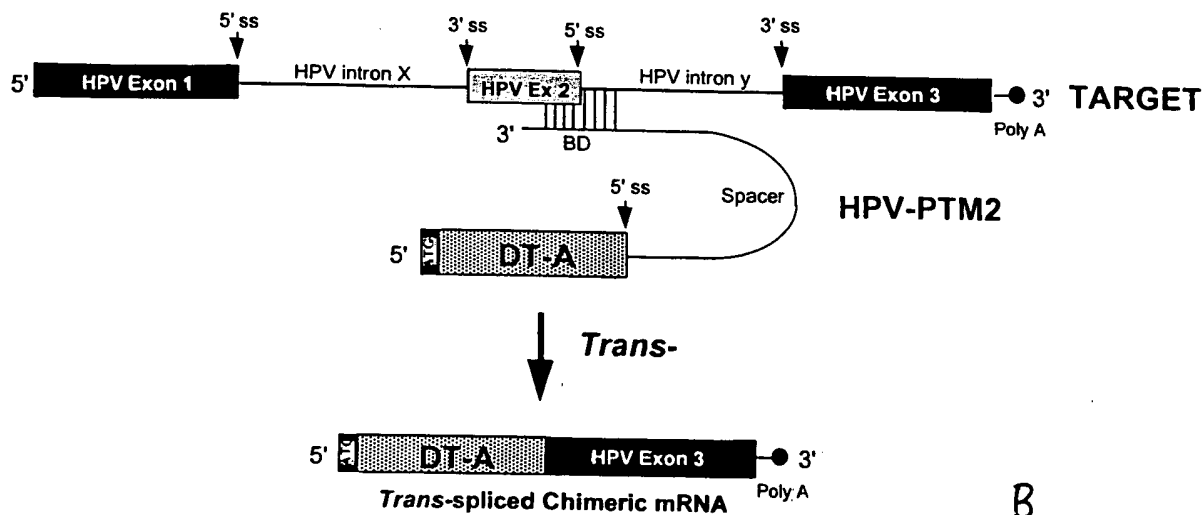


FIGURE 66

HPV-PTM3 (For Internal exon replacement)

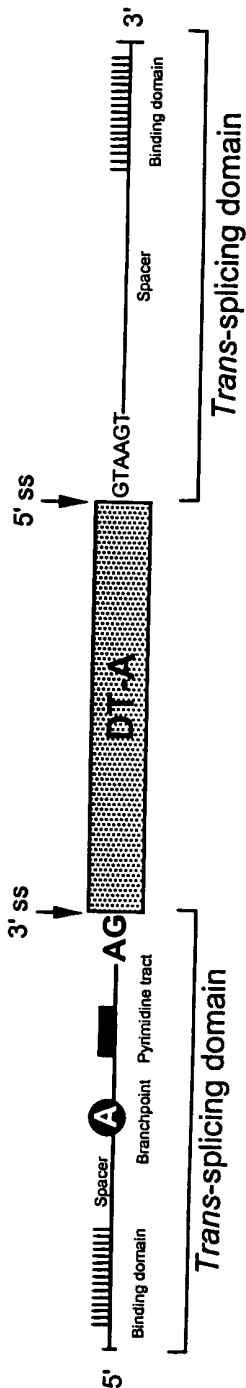


FIGURE 67